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Efeito da alta-pressão na fermentação de bactérias malolácticas

High pressure effect on malolactic bacteria growth and metabolism

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro

À minha família e amigos

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Palavras-chave *Oenococcus oeni*, fermentação maloláctica, ácido L-málico, ácido L-láctico, ácido D-láctico, alta-pressão

Resumo

O *Oenococcus oeni* é uma espécie de bactéria de elevado interesse, nomeadamente enológico, porque é capaz de realizar a fermentação maloláctica (desacidificação do vinho) e reúne as condições essenciais para poder sobreviver às condições adversas do vinho. Por outro lado ao alta-pressão é uma tecnologia com grande potencial para explorar novas e promissoras aplicações na biotecnologia.

Neste trabalho pretendeu-se avaliar o efeito de um tratamento de alta-pressão no metabolismo desta bactéria, nomeadamente na descarboxilação do ácido L-málico, no metabolismo de açúcares e no crescimento bacteriano.

O tratamento de 50 MPa durante 8 h e 100 MPa durante 0.5 h não resultaram em alterações significativas no metabolismo das bactérias. O tratamento de 100 MPa, durante 8 e 60 h resultaram numa redução da quantidade de ácido L-láctico produzido, propondo-se que também foi produzido ácido D-láctico a partir do ácido L-málico. O tratamento de 300 MPa durante 0.5 h resultou na completa inactivação das bactérias.

Assim conclui-se que alta-pressão é uma tecnologia que permite a alteração do metabolismo, nomeadamente a modificação da especificidade da enzima maloláctica, e a inactivação de *Oenococcus oeni*.

Keywords *Oenococcus oeni*, malolactic fermentation, L-malic acid, L-lactic acid, D-lactic acid, high hydrostatic pressure

Abstract

The *Oenococcus oeni* is a bacterial species with high interest, especially winemaking, because it is able to carry out malolactic fermentation (wine desacidification) and has the essentials conditions to be able to survive in the wine harsh conditions. On the other hand the high-pressure is a technology with great potential to explore new and promising applications in biotechnology.

In this work the aim was to evaluate the effect of high pressure treatments in the metabolism of the bacteria, especially in the decarboxylation of L-malic acid, in the sugars metabolism and bacterial growth.

The treatment of 50 MPa during 8 hours and 100 MPa during 0.5 h did not result in significant alterations in the bacteria metabolism. The treatment at 100 MPa during 8 and 60 h resulted in a reduction of the amount of L-lactic acid produced. It was proposed that was also produced D-lactic acid from L-malic acid. The treatment of 0.5 MPa during 300 h resulted in complete inactivation of bacteria.

It is concluded that high pressure is a technology that allows the alteration of the metabolism, particularly change of malolactic enzyme specificity, and inactivation of *Oenococcus oeni*.

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CHAPTER I

GENERAL INTRODUCTION

1.1 Malolactic fermentation in wine

1.1.1 History

The occurrence of a "second" fermentation in young wines, starting with the rise in temperatures during late spring (usually while the vines were flowering), is described in 1837, by Freiherr von Babo in his book, "A Short Education on Suitable Treatments of Vinificated Juices". This "second" fermentation was responsible for renewed turbidity in the new wines and liberated CO_2. Von Babo related this activity to "the melting of the grease" of the alcoholic fermentation and suggested an immediate racking into a new barrel with sulfur dioxide, fining and temperature reduction, followed by a second racking and stabilization with another addition of sulfur dioxide (1).

Louis Pasteur, in 1866, isolated the first bacteria from wine, during his studies on wine spoilage and began his "Études sur le vin" (2). He was also responsible for the general opinion that all bacteria in wine are spoilage microorganisms. The acids content reduction observed in wine was still related to precipitation of tartaric acid, although in 1891, Hermann Müller-Thurgau (3) had already postulated that the acid reduction could be due to bacterial activity. His theory was confirmed by Koch (4) and Seiffert (5) in 1898 and 1901. In 1913, Müller-Thurgau and Osterwalder, with their epoch-making investigation into lactic acid bacteria in wine (6), explained the bacterial degradation of malic acid to lactic acid and CO_2 according to the formula (Equation 1):

$$C_4H_6O_5 = C_3H_6O_3 + CO_2 \qquad \textbf{Equation 1}$$

They called this phenomenon "biological deacidification" or "malolactic fermentation", and *Bacterium gracile* was described as the agent responsible. In the 1950s, the application of new enzymatic methods helped explain the enzymatic reactions that occur during the degradation of malic acid (7). Improved analytical methods applied by Radler (8), Peynaud (9), Beelman (10) and Kunkee (11) resulted in a better understanding of the complex nutrient demands of the wine lactic acid bacteria, since degradation of malic acid alone gives only a minor energetic advantage to the bacterial strain (12).

Since these early findings, research on lactic acid bacteria has progressed. The name *Bacterium gracile*, frequently used in the past as the name of the organism that caused malolactic fermentation, was revised. Findings by Vaughn (13) and Radler (8) showed that the lactic acid bacteria in grape must and wine belong to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and, more recently, *Oenococcus* (14).

The following time-line resumes the history of the malolactic bacteria knowledge (Fig 1).

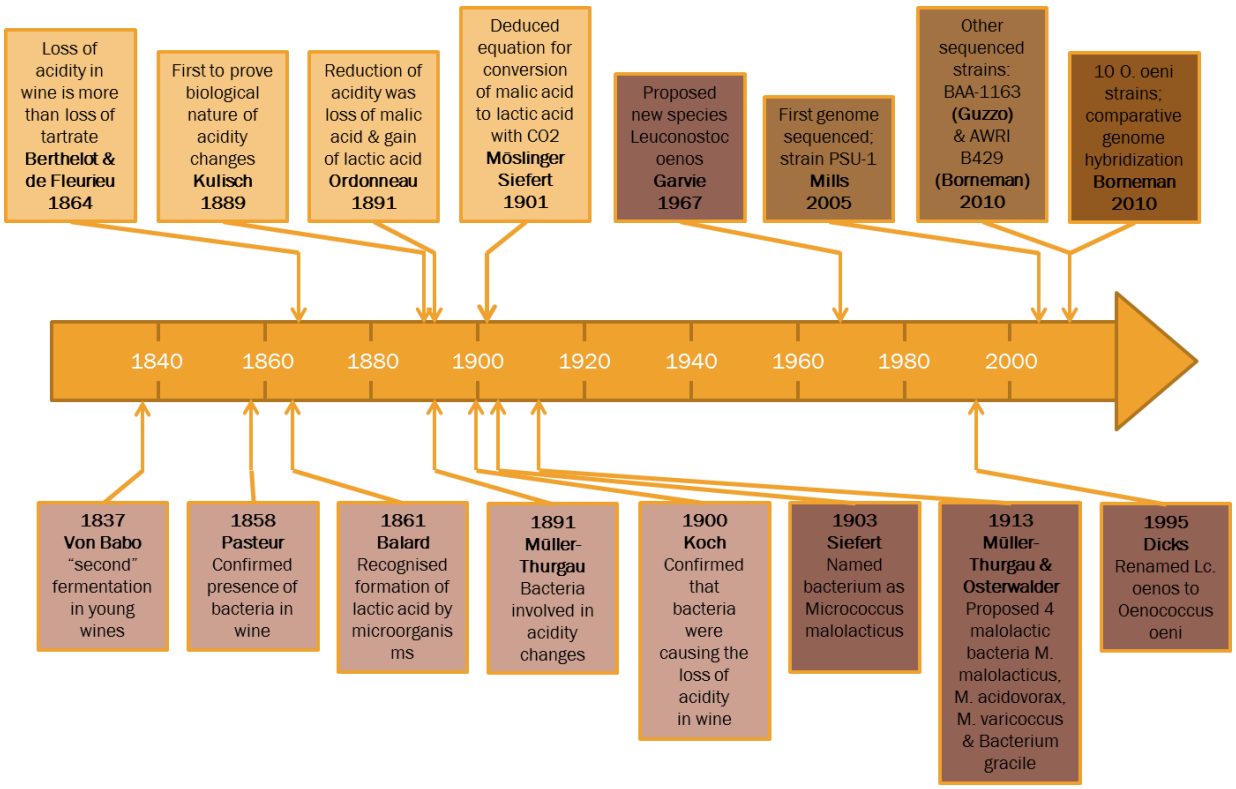


Figure 1 – Summary time-line of the identification of bacteria in wine, elucidation of their role in winemaking, classification and genome analysis (adapted) (15)

1.1.2 Malolactic microorganisms

1.1.2.1 Lactic acid bacteria

Lactic acid bacteria are found naturally on grapes, leaves, soil and equipment surfaces and have the ability to grow on a variety of sources, including grape juice. The most common lactic acid bacteria belong to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (16, 17). These bacteria are generally microaerophillic, require carbohydrates, and must be supplied with amino acids and vitamins in order to proliferate (18, 19).

Typically, lactic acid bacteria identified in grape musts are present at approximately 10^4 cells per mL. The majority of these bacteria is not tolerant towards the changing environmental conditions associated with winemaking and disappears during alcoholic fermentation. However, many species are able to survive, in particular *Oenococcus oeni*, which can withstand alcoholic fermentation (20) and is often found in wines with a pH below 3.5. Wines exhibiting a pH greater than 3.5 are capable of supporting a broader range of species including *Lactobacillus brevis*, *Lactobacillus buchnerii*, *Lactobacillus casei*, *Lactobacilllus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus fructivorans*, *Lactobacillus hilgardii*, *Lactobacillus jensenii*, *Lactobacillus kunkee*i, *Lactobacillus nagelii*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus desidiosus*, *Pediococcus parvulus*, *Pediococcus damnosus* (formerly *Pediococcus cerevisiae*), *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, *Leuconostoc gracile* and *Oenococcus oeni* (14, 19, 21-39).

Regardless of the species of lactic acid bacteria, the main significance of these organisms in wine production is their ability to conduct malolactic fermentation. This fermentation is characterized by the degradation of L-malic acid to L-lactic acid and CO_2, a process which decreases the acidity of wine. However, malolactic fermentation not only represents a biological deacidification process, it also exerts a significant impact on the organoleptic aspects of wine. These sensory effects can be positive or negative, depending on the bacterial species, and, more specifically, the strain of lactic acid bacteria employed to conduct the malolactic fermentation (16, 40, 41).

Lactic acid bacteria strains that produce particularly favourable characteristics in wine, and hence are more desirable to perform the malolactic fermentation, are often termed “malolactic bacteria”. lactic acid bacteria strains that negatively influence the final product may cause a range of undesirable changes to wine sensory properties, altered wine colour, and may even lead to the generation of biogenic amines (16).

Given the important role of the organism employed for malolactic fermentation, it is an increasingly common practice to “seed”, “implants” or “inoculate” a fermentation with a known malolactic bacteria strain or a mixture of strains, rather than depend on the naturally occurring flora. The advantage of inoculating is that the time and the extent to which malolactic fermentation occurs can be controlled and the quality of the final product can be predicted (42).

As previously described, there are many organisms that are capable of performing malolactic fermentation in wine. Species of *Lactobacillus* or *Pediococcus* may conduct

malolactic fermentation, especially in wine exhibiting a pH higher than 3.5, but usually result in non-acceptable wines. These genera are poorly tolerant to low pH and produce undesirable flavours as well as high levels of acetic acid. An exception to this rule is *Lactobacillus plantarum*, which does not form a significant amount of acetic acid and has been suggested as a suitable candidate for malolactic fermentation (43). However, due to its moderate tolerance to ethanol, it requires inoculation into the must prior to alcoholic fermentation (44). Other species of *Lactobacillus*, for example *Lactobacillus hilgardii*, *Lactobacillus casei* and *Lactobacillus brevis*, are more ethanol tolerant but produce a different balance of metabolic products. Although it is possible to perform malolactic fermentation using these strains, they may produce acetic acid, and so their use as commercial starters is limited (45).

Some yeast species are also able to degrade malic acid through an alternative pathway. Such *Saccharomyces* strains can catabolize significant amounts of malic acid and convert it to ethanol rather than to lactic acid. This conversion has been termed the maloethanolic fermentation and implies that a potentially greater reduction of wine acidity may be achieved.

1.1.2.1.1 Taxonomy of wine lactic acid bacteria

Due to the highly selective environment of different juices and wines, only a few types of lactic acid bacteria are able to grow in wine (9, 19, 46).

The following general description is valid for all wine lactic acid bacteria:

• Gram-positive;

• Non-mobile and non-sporulating;

• Facultative anaerobes;

• Chemoorganotrophic metabolism – They require a rich medium and fermentable sugars;

• Optimum growth temperature of 20°-30°C.

In addition to their coccid (round) or rod-like shapes, homofermentative or heterofermentative sugar metabolism is a deciding factor in their classification. Homofermentative bacteria produce lactic acid from glucose and/or fructose (9, 19, 46). Heterofermentative lactic acid bacteria produce carbon dioxide, ethanol and acetic acid, as well as lactic acid from the same carbohydrates. The *lactobacilli* can possess both types of carbohydrate metabolism, and they are divided into three groups (9, 19, 46):

- Strict homofermenters – This group has never been detected in wine.

- Facultative heterofermenters – One molecule of glucose is converted into two molecules of lactic acid. Pentoses are fermented into lactic acid and acetic acid.
- Strict heterofermenters – They ferment glucose into lactic acid, acetic acid, ethanol and CO_2. Pentoses are fermented into acetic acid and lactic acid.

This classification is likely to be modified because of progress in the identification of new bacterial isolates from wine, as well as advances in the molecular biological techniques used to identify the isolates. For example, in 1995 Dicks *et al.* (14) showed that *Leuconostoc oenos* was distinguished from other *Leuconostoc* species not only by its growth in acidic media, its requirement for a tomato juice growth factor and its carbohydrate fermentation pattern, but also by DNA-DNA hybridization and numerical analyses of soluble cell protein patterns. Phylogenetic studies, in particular those involving 16S and 23S rRNA sequences, have revealed a distinct subline of *Leuconostoc oenos* that is separate and distinct from other *Leuconostoc* species as well as other lactic acid bacteria in general. This subline is genotypically homogeneous and would form a distinct grouping in *Leuconostoc oenos*. Therefore, it was assigned to a new genus named *Oenococcus oeni* (14).

1.1.2.1.2 *Oenococcus oeni*

Oenococcus oeni is currently the preferred bacterial species to conduct malolactic fermentation, rather than yeast or other lactic acid bacteria, and the precise reasons for this are described in greater detail in the following section. It is important to note that the reluctance of the wine industry to employ alternative bacterial species may be in part due to the lack of availability of alternatives. It is likely that strains of *Pediococcus* and *Lactobacillus* may be isolated in the future and prove particularly adept at performing malolactic fermentation. The rational for the popularity of *Oenococcus oeni* is that this organism is particularly adept at withstanding the harsh environment of wine and is capable of quickly converting malic acid to lactic acid. In addition, despite being genetically homogeneous, there is a significant degree of phenotypic heterogeneity within strains of *Oenococcus oeni*. The consequence of this is that different strains of *Oenococcus oeni* can have notably different effects on the final product, and it has been demonstrated that some strains are more beneficial to the properties of wine than others (28, 47).

Oenococcus oeni, firstly known as *Leuconostoc oenos* (48), is a facultative anaerobe and can be propagated in a variety of low pH media (pH 4.2-4.8) supplemented with tomato juice or grape juice. Nutritional requirements are complex and have been described in detail by Henick-Kling (18). A source of carbon (derived from sugars), nitrogen (derived from free amino acids or short peptides), vitamins (nicotinic acid, thiamine, biotin and pantothenic acid), mineral ions (Mn^{2+}, Mg^{2+}, K^{+} and Na^{+}) and purine derivatives (guanine, adenine, xanthine and uracil) are all required for optimum growth (18). *Oenococcus oeni* cells are spherical and occur in chains when grown on solid media. Growth is generally slow and can take from 5 to 7 days to form visible colonies at incubation temperatures between 20°-30°C (49).

Although previously grouped with the *Leuconostoc* species, DNA analysis of *Oenococcus oeni* strains has placed them in a group that is clearly distinguishable from the *Leuconostoc* species. As a group, *Oenococcus oeni* strains are genetically homogeneous, as demonstrated by the analysis of soluble cell protein patterns, DNA-DNA hybridization and sequence analysis of the 16S-23S rDNA intergenic spacer region (50-54). It has been suggested that this homogeneity may be the result of a clonal lineage and a specialized relationship with viticulture and wine production over many years (53). Genetic homogeneity is not manifested in the effect of *Oenococcus oeni* on wine, and the particular strain utilized can have markedly different effects on the characters of the final product. Phenotypic studies of *Oenococcus oeni* have shown considerable diversities in terms of lactate dehydrogenases, carbohydrate fermentation and cellular fatty acids (55, 56). Interestingly, Guerrini *et al.* (57) have been able to highlight phenotypic and genotypic specificity for several wine-producing areas, indicating the natural evolution of strains from different regions of the world.

It is widely believed that *Oenococcus oeni* represents the best candidate to conduct malolactic fermentation because of its resistance to a variety of environmental stresses, in particular the acidic conditions and the high alcohol levels which are typical of wine. Inoculating wine with carefully selected strains of *Oenococcus oeni* has the advantage of enabling the producer to have more control over malolactic fermentation. In addition, employing a specific strain of *Oenococcus oeni* allows the winemaker to ensure that particular characteristics are produced in the final product, thus creating wines that are more distinctive and characteristic (58).

Although a single bacterial strain is generally employed, in some instances a mixture of strains may be used in the inoculum. This procedure can not only produce

certain preferred characteristics in the wine, but is also capable of maximizing the chances of bacterial survival if a bacteriophage is encountered in the wine (59).

1.1.2.1.3 Lactic acid bacteria growth influencing factors in wine

During the production of wine, a series of highly dynamic systems are formed and the interaction between components within this system can influence the success of the fermentation. The composition of the wine, the factors associated with the method of vinification and the interrelationships between lactic acid bacteria and other microorganisms present can affect the survival and growth of lactic acid bacteria in wine and therefore influence malolactic fermentation. However, environmental conditions such as pH, temperature, alcohol level, nutritional status and sulphur dioxide (SO_2) may also play a significant role (16, 19, 25).

The specific factors will be discussed in detail, but with regard to wine composition, pH is one of the most important parameters affecting the behavior of lactic acid bacteria in wine, and it also exerts a selective action on which lactic acid bacteria strains will be present. Wine pH influences which bacterial species will grow, the viability and rate of growth of the lactic acid bacteria, the rate of malic acid degradation and the metabolic behavior of the bacterial species. The minimum pH at which bacterial growth can occur in wine is approximately 2.9-3.0. Bacterial growth is faster and malolactic fermentation is completed earlier as the pH increases above 3.0. Although a pH of 6.3 is optimum for the activity of the malolactic enzyme, degradation of malic acid by non-growing cells of *Oenococcus oeni* is most rapid at lower pH values (4.7) due to an increase in intracellular pH (4.0). It is widely accepted that in terms of initiation and completion of malolactic fermentation, a pH of approximately 3.4 is the most desirable (25).

As previously described, alcohol tolerance is an important characteristic of many lactic acid bacteria, and resistance to alcohol varies among them. Most strains are not capable of proliferating in wines with an ethanol concentration greater than 15% but some have been observed to grow in the presence of 20% ethanol (19).

The optimum growth temperature for lactic acid bacteria, mesophyllic bacterias, is between 25° and 35°C and the rate of malate degradation by non-growing cells is highest at approximately the same temperatures. The rate of growth of malolactic bacteria and the speed of the malolactic fermentation are reduced by low temperatures.

This can be problematic, particularly during the production of white wines, which tend to be fermented at lower temperatures (18, 60).

With respect to interactions with other wine organisms, mixed cultures of microorganisms introduce the possibility of antagonistic and synergistic relationships, but, in some minor cases, may have no effect. In winemaking, there is the possibility of the interaction of lactic acid bacteria with yeast, fungi, acetic acid bacteria and bacteriophage, as well as interactions between species and strains of lactic acid bacteria (61). The antagonistic effect of yeasts has been explained by the competition for nutrients and the production of substances that inhibit bacterial growth, such as SO_2 or medium-chain length fatty acids. On the other hand, yeasts may support the growth of lactic acid bacteria in wine as well as stimulate the malolactic fermentation. During extended lees contact with wine, the process of yeast autolysis releases vitamins and amino acids into the wine. This results in nutrient enrichment and subsequent stimulation of the malolactic fermentation. However, Costello reported that growth of *Pediococcus ssp* was supported by the rapid cell death of *Oenococcus oeni*, and under high pH conditions the early growth of *Lactobacillus brevis* completely inhibited the growth of *Oenococcus oeni* (62).

The presence of bacteriophages, viruses able to kill bacteria, can also inhibit malolactic fermentation in wines. Strains of bacteriophage that can resist adverse conditions and induce cell lysis of malolactic bacteria have been isolated, and they are capable of disrupting the population dynamics of malolactic fermentation. To counter the action of bacteriophage, strains of *Oenococcus* resistant to certain bacteriophage can be selected. Alternatively, a mixture of bacterial strains may be employed as malolactic fermentation starters to maximize the potential for the culture to survive. Although the presence of bacteriophage can seriously affect the quality of wine, they tend to be inhibited during active growth of malolactic bacteria and their potential effect can be minimized by ensuring conditions that favour the growth of the desirable malolactic organism (16, 18, 63, 64).

Sulphur dioxide (SO_2) strongly inhibits the growth of lactic acid bacteria, but the sensitivity of lactic acid bacteria to SO_2 varies. Sulphur dioxide is more inhibitory at low pH. Growth and malolactic fermentation by lactic acid bacteria are increasingly inhibited at alcohol concentrations above 6%, with 14% (v/v) being the upper limit tolerated by most strains. With regard to winery practices, juice and wine clarification can remove a large portion of the lactic acid bacteria and also reduce the incidence of

bacterial growth and its effect on wine quality. During clarification, some nutrients and suspended particles that are stimulatory to bacteria growth will be removed. Wines made by thermo-vinification have been reported as being less suitable for malolactic fermentation. The timing of the inoculation of malolactic bacteria also influences the kinetics of malolactic fermentation (18).

1.1.2.2 Other microorganisms

Maloethanolic fermentation is distinct from malolactic fermentation, although the end result is comparable in terms of the degradation of malic acid. Interestingly, the transformation of a *Saccharomyces cerevisiae* strain with bacterial malolactic genes to enable it to perform an enhanced maloethanolic fermentation has also been performed. Unfortunately, in these instances the transformed yeast strains were observed to exhibit a low malate catabolic activity (65, 66).

However, complete malolactic fermentation has been achieved using *Saccharomyces cerevisiae* strains co-expressing the genes *mles* and *mae1* which code for the *Lactococcus lactis* malolactic enzyme and the *Schizosaccharomyces pombe* malate permease, respectively, both of which are under the control of yeast promoters. Despite this discovery, the effect of such strains on the flavour profile of the final product has yet to be elucidated. Furthermore, it is unlikely that these strains will be utilized for commercial production of wine due to the current perception of consumers towards the use of genetically modified organisms (67).

Schizosaccharomyces pombe strains have also been employed for malolactic fermentation, but have been found to produce a number of undesirable flavours in wine, including hydrogen sulphide. *Schizosaccharomyces pombe* strains are also sensitive to ethanol, resulting in a requirement to inoculate prior to alcoholic fermentation (68-71).

1.1.3 Malolactic fermentation by lactic acid bacteria:

1.1.3.1 General metabolic properties

Lactic acid bacteria constitute a ubiquitous group of bacteria that occur in a range of environments, including many foods and beverages. Importantly, these bacteria are primarily noted for their ability to produce lactic acid from a fermentable carbohydrate source. Lacking heme-linked cytochromes and catalase, lactic acid bacteria obtain energy from carbohydrates by fermentative metabolism (72).

The lactic acid bacteria can be broadly classified as either homofermentative or heterofermentative according to the types of end-products that are produced from the fermentation of glucose. Homofermentative lactic acid bacteria, including the *pediococci* and some of the *lactobacilli*, utilize the glycolytic Embden-Meyerhof-Parnas pathway to convert the hexose sugar – glucose – mainly to lactic acid. In this pathway, two moles of lactic acid and two moles of ATP are produced for each mole of glucose fermented (Fig. 2).

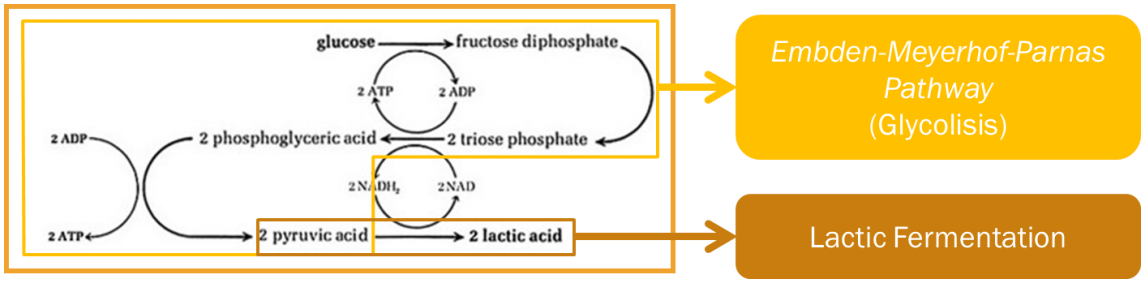


Figure 2 – Scheme of the Embden-Meyerhof-Parnas pathway and lactic fermentation

On the other hand, the heterofermentative *lactobacilli* and the *leuconostocs* lack some key enzymes of the Embden-Meyerhof-Parnas pathway and ferment hexose sugars by the phosphoketolase pathway. In this pathway, equimolar concentrations of lactic acid, CO_2 and acetic acid or ethanol can be produced from one mole of glucose, with a concomitant energy gain of one mole ATP (Fig. 3). The oxidation/reduction potential (redox) of the system also affects the ratio of ethanol/acetic acid produced, with aerobic conditions favouring the formation of acetic acid, and anaerobic conditions favouring the production of ethanol (72, 73). Depending on the species or the genus of lactic acid bacteria involved, the isomers of lactic acid produced from the fermentation of carbohydrates can be either L(+), D(-) or a combination of both the L(+) and D(-) forms (72, 74). For example, *Leuconostocs*, including *Oenococcus oeni*, produce the D(-)-lactic acid isomer from the fermentation of hexose sugars. Differently, the

decarboxylation of L(-)-malic acid in the malolactic fermentation yields only the L(+)-lactic acid isomer (72, 74).

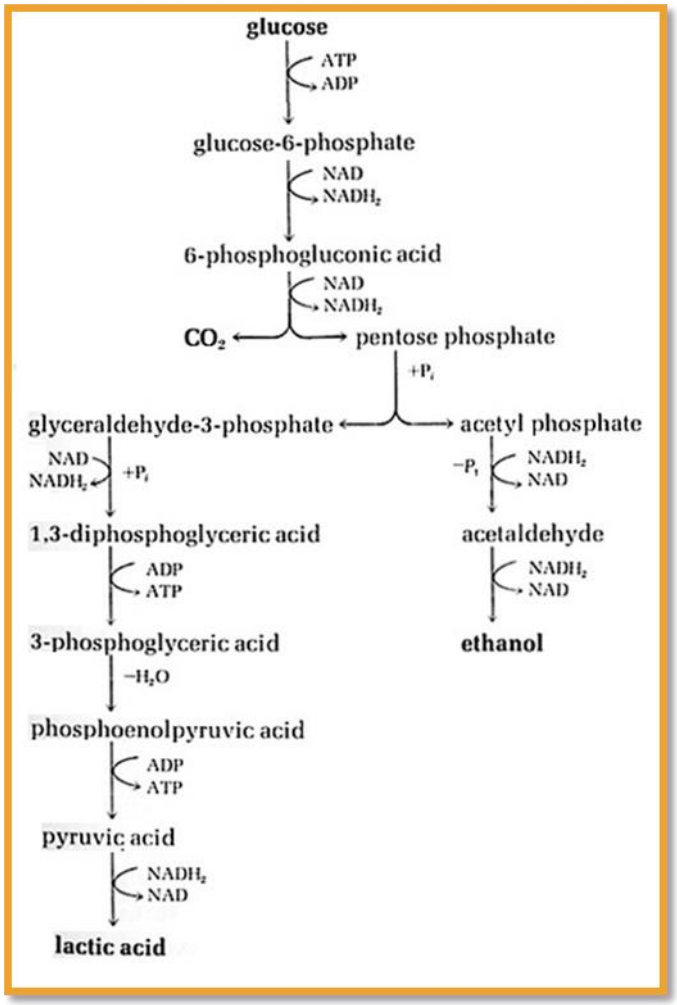
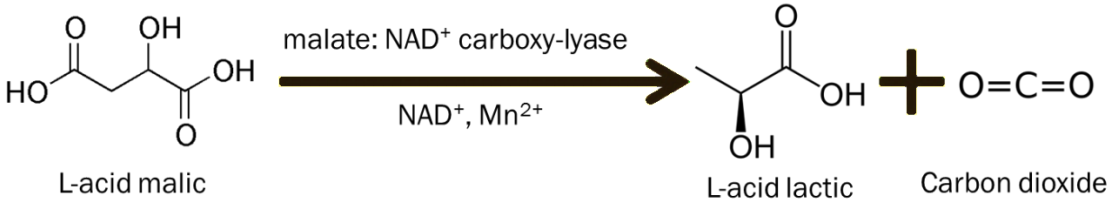


Figure 3 – Scheme of the phosphoketolase pathway

Overall, the lactic acid bacteria group can utilize a wide range of carbohydrates, including the hexoses (glucose, fructose, mannose and galactose), as well as other pentoses, polyols and oligosaccharides. This capability is dependent on the species and strains involved, as well as the pH of the medium. Moreover, since malic acid cannot be used by wine lactic acid bacteria as a sole carbohydrate source, the availability and utilization of fermentable carbohydrates in wine by lactic acid bacteria is essential to enable the onset of bacterial growth and the occurrence of malolactic fermentation. Further, recent studies have clearly demonstrated that grape-derived phenolic glycosides also significantly stimulate the growth of *Oenococcus oeni* in a synthetic wine medium (72, 74).

1.1.3.2 Malolactic reaction

Overall, three main pathways have been proposed for the degradation of L-malic acid to L-lactic acid by lactic acid bacteria during malolactic fermentation. The first involves the activity of three separate enzymes, malate dehydrogenase, oxaloacetate decarboxylase and L-lactate dehydrogenase, and proceeds via the intermediates oxaloacetic acid and pyruvic acid. A second mechanism proceeds via pyruvic acid and utilizes a combination of malic enzyme and lactate dehydrogenase. It was not until the 1970's that the enzymatic basis for this reaction was more fully elucidated in wine malolactic bacteria, specifically *Leuconostoc oenos* (*Oenococcus oeni*) ML34, by Kunkee and Morenzoni (75, 76). This work revealed that a single enzyme, commonly known as the "malolactic enzyme," exhibits two separate enzyme activities which act simultaneously on L-malic acid. The predominant "malolactic activity" of this enzyme (malate: NAD^+ carboxy-lyase) catalyzes the direct conversion (decarboxylation) of the dicarboxylic acid L-malic acid to the monocarboxylic acid L-lactic acid, and requires NAD and Mn^{2+} as co-factors (Equation 2) (75, 76).



Equation 2

The malolactic enzyme from *Leuconostoc oenos* (*Oenococcus oeni*) has a molecular mass of 138,000 and consists of two identical subunits, each with a molecular mass of 65,500 (77).

1.1.3.3 Biological and energetics role

There has been considerable investigation in recent decades concerning the seemingly obscure benefit of the malolactic conversion to the bacterial cell. The initiation of malolactic fermentation in wine usually occurs after lactic acid bacteria have grown beyond a viable cell population of approximately 10^6 CFU/mL. Although providing deacidification and an accompanying increase in pH of up to approximately 0.2 pH units, the malolactic conversion itself appears energetically slightly favourable to lactic acid bacteria. It yields little free energy (ΔG = -8.3 kJ/mole), proceeds without

the formation of free intermediates and does not yield biologically available energy in the form of ATP. Further, although NAD is an essential co-factor, it does not serve an oxidation/reduction function as there is no net change in redox state (74, 77-80).

In overall terms, malolactic fermentation is not a true fermentation. In addition to supplying little energy for cell growth, it also does not supply a source of carbon for the biosynthetic reactions that are essential for cellular development. Nevertheless, the presence and utilization of malic acid appreciably stimulates the initial growth rate of malolactic bacteria, yet the resulting increase in pH that is associated with malolactic fermentation does not fully account for this stimulatory effect (74, 78, 79).

Although the conversion of L-malic acid to L-lactic acid by the malolactic enzyme is energetically slightly favourable, the malolactic fermentation has, in fact, been shown to provide energy in the form of ATP to the bacterial cell. This is accomplished by a chemiosmotic mechanism which generates a proton motive force (Δp) across the cell membrane. In this model, the malolactic fermentation proceeds in three stages.

In the first step, entry of L-malic acid into the bacterial cell is facilitated by a specific transport enzyme (Fig. 4).

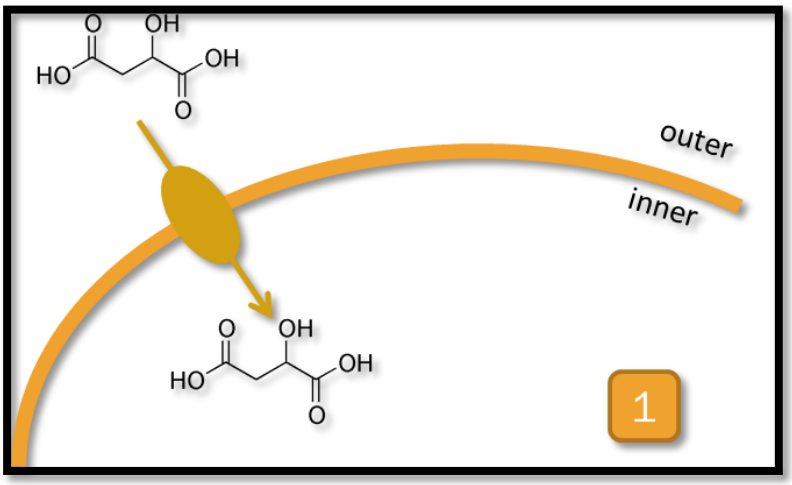


Figure 4 – Scheme of the first step of the energetic role

In the second step, L- malic acid is decarboxylated within the cell by the malolactic enzyme, yielding L-lactic acid and CO_2, which then increases the intracellular pH (Fig. 5).

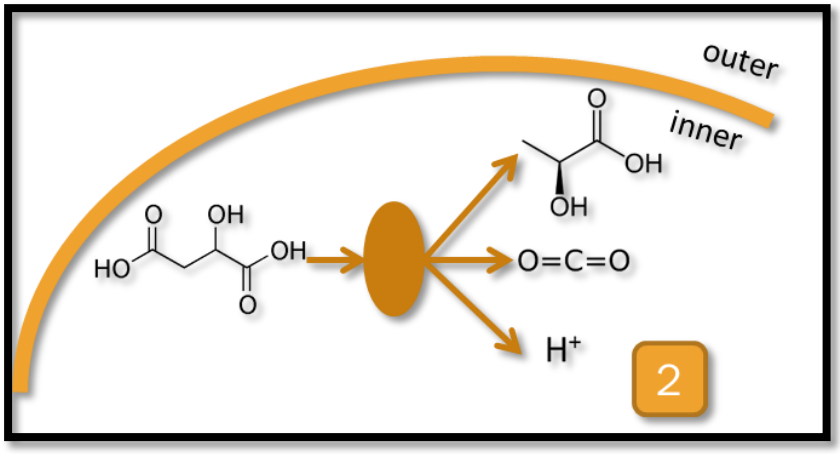


Figure 5 - Scheme of the second step of the energetic role

In the final stage, L-lactic acid and CO_2 are expelled from the cell. For every molecule of lactic acid that leaves the cell, one proton is also translocated outside of the cell. This establishes a proton gradient across the cell membrane between the cytoplasm and the surrounding medium. This gradient, combined with a specific ATPase in the cell membrane, facilitates the generation of energy available for transport processes in the form of ATP. The synthesis of one ATP requires the entry of three protons through the membrane-bound ATPase (Fig. 6) (80-84).

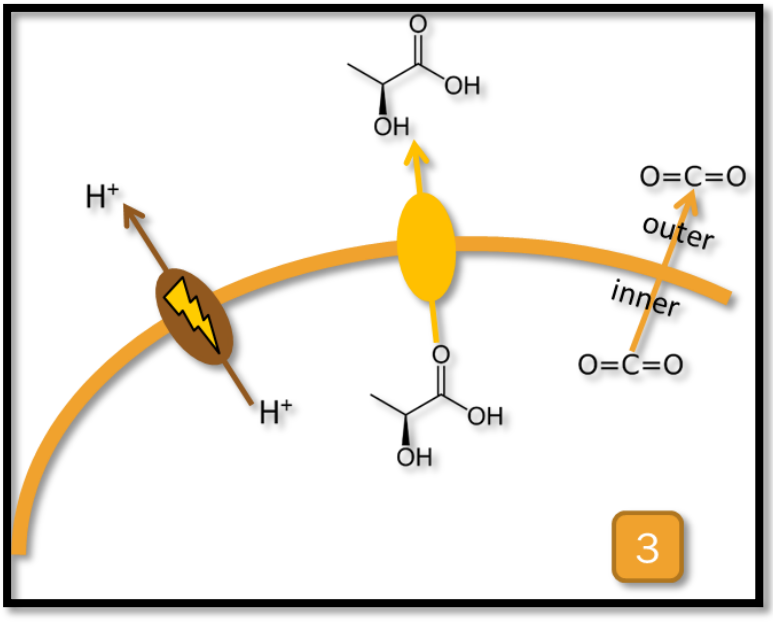
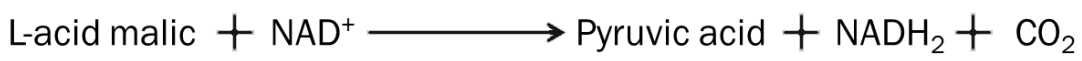


Figure 6 - Scheme of the final step of the energetic role

Malic and citric acids do not serve as the sole energy sources for the growth of lactic acid bacteria (85). Consequently, malolactic bacteria require sugars as a carbon source. However, under conditions of limiting sugar availability or of low pH,

which inhibit sugar metabolism, energy (ATP) generated from malolactic fermentation is beneficial to cell growth (80). Another, but minor (<1%), activity of the malolactic enzyme has also been suggested to stimulate the metabolic activity and initial growth rates of wine lactic acid bacteria. This secondary malolactic enzyme activity catalyzes the next reaction (Equation 3) (75).



Equation 3

The very small amounts of pyruvic acid and $NADH_2$ generated by this secondary malolactic activity are considered to stimulate the initial stages of glucose metabolism and initial growth rates through the provision of hydrogen acceptors (74, 77).

In addition to the role of malolactic bacteria in conducting malolactic fermentation, certain yeasts, including *Schizosaccharomyces pombe*, are also capable of catabolizing malic acid. However, this metabolism is not a true malolactic fermentation since malic acid is metabolized to ethanol (69, 86).

Despite its potential for wine deacidification, drawbacks to using yeast maloethanolic fermentation by species of *Schizosaccharomyces spp.* include the formation of undesirable flavour compounds, such as hydrogen sulphide (16, 68-70, 87).

1.1.3.4 Influence of malolactic fermentation on wine composition

1.1.3.4.1 Acidity reduction

For each molecule of L-malic acid catabolized to the weaker L-lactic acid through the malolactic fermentation, there is a stoichiometric loss of a carboxyl group and the corresponding reduction in wine acidity. In addition to the dependency of such effects on the initial concentration of malic acid, the actual changes in wine acidity and pH attributable to the malolactic fermentation depend on other factors, including the buffering capacity of the wine as well as the initial pH (74). In general, the overall decrease in wine acidity resulting from malolactic fermentation can vary from 0.1%-0.3%, and pH may rise by 0.1-0.3 pH units (16). Wines produced from grapes cultivated in cool climate viticultural areas contain a naturally high level of acidity of up to approximately 8 g/L malic acid, and are considered to benefit from such an acid

reduction. On the other hand, wines produced from grapes grown in warm to hot regions have lower acidity (5.0-6.5 g/L), and a further reduction in acidity from malolactic fermentation can have a negative impact on wine quality, causing a flat taste and a greater predisposition to bacterial spoilage (88). Nevertheless, malolactic fermentation can be desired in such wines to confer a degree of biological stability and/or to impart flavour complexity, necessitating the use of acidulants to adjust wine acidity and pH to acceptable levels after malolactic fermentation. The increase in wine pH accompanying malolactic fermentation can also influence wine colour (88).

1.1.3.4.2 Flavour changes

Although there has been conjecture over the contribution of malolactic fermentation to wine sensory properties (16), more recent research has provided greater insight into specific sensory changes associated with the growth and metabolic activity of malolactic bacteria in wine. It is clear that different strains of malolactic bacteria may increase or decrease the intensity of certain wine aroma and flavour attributes, and those changes are strain dependent (89). In addition to deacidification, flavour attributes imparted by malolactic fermentation can be described as buttery, lactic, nutty, yeasty, oaky, sweaty and earthy. malolactic fermentation may also impact fruity and vegetative aromas, as well as the mouthfeel of wine (80, 84, 90). Mechanisms by which malolactic bacteria can influence wine flavour may include (removal of existing flavour compounds by metabolism and adsorption to the cell wall, (production of new bacterial-derived flavour compounds from the metabolism of sugars, amino acids and other substrates, and metabolism and modification of grape and yeast-derived secondary metabolites to end-products having greater or lesser sensory impact (89).

Some of these flavour-active compounds, including acetaldehyde, acetic acid, diacetyl, acetoin, and 2,3-butanediol. Diacetyl, acetoin and 2,3-butanediol originate from the bacterial consumption of citric acid and are of considerable importance to the flavour profile of wine. In lower concentrations, these compounds are felt to add complexity to the wine flavour. At concentrations in excess of 5 mg/L, diacetyl can be overpowering, giving the wine a distinct buttery/nutty flavour. Depending on the pH and the oxidation-reduction potential of the wine, acetic acid can be another metabolite from the degradation of citric acid by malolactic bacteria. Increased levels of volatile esters, ethyl lactate and higher alcohols were also reported in

wines undergoing malolactic fermentation (91). Henick-Kling has described the flavour contributions of individual strains of malolactic bacteria (92).

Importantly, the net impact of malolactic fermentation on wine sensory properties will depend on factors such as bacterial strain characteristics, varietal aroma intensity of the wine and vinification techniques employed (84).

1.2 High pressure processing

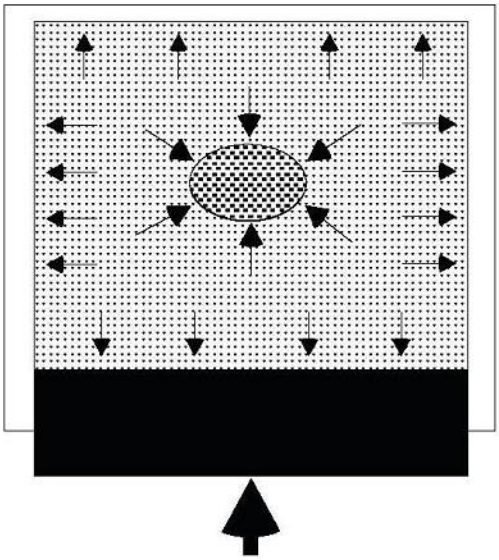


Figure 7 - Schematic representation of hydrostatic pressure (93)

Pressure is defined as the force per unit area applied in a perpendicular direction. Mathematically (Equation 4):

$$p = F/A$$

Equation 4

Where p is the pressure, F the normal force applied to a surface and A the surface's area. The SI unit of the pressure is called pascal (Pa) (newton per square metre), but this unit is very small, so in high pressure studies the unit commonly used is the Megapascal (MPa) (1MPa= 106Pa) (Fig. 7).

Just like temperature, pressure is a parameter characteristic of Biosphere. In the terrestrial habitats, where pressure value is close to one bar or lower, account for less than 1% of the total volume of the biosphere. Whereas the ocean, which cover approximately 70% of the surface of the Earth, have an average depth of 3800 m and consequently an average pressure of 381 atm (38.5 MPa), however the greatest depth in the oceans, the "Challenger Deep" in the Marianas Trough, is near 11000 m (94). Approximately 79% of the volume of the marine component of the biosphere lies below 1000 m (94).

Moreover, the discovery of piezophile (or barophiles) and thermophile micro-organisms has led some recent studies to consider that pressure, and particular hydrothermal processes, could be at the origin of life (95-98).

The pressure can profoundly influence molecular systems. However, compared to heat, the effects of high pressure on living systems and biomolecules have historically not received the same attention (95-98).

1.2.1 Biological effect

While piezophile microorganisms are adapted to high pressure conditions, the mesophilic microorganisms will induce its stress response to these conditions, because pressure is able to influence most biochemical reactions, since they often involve a change in volume. Volume increasing reactions will tend to be inhibited by pressure, while reactions leading to a decrease in volume will tend to be promoted; such is postulated in the Le Chatelier's principle (99, 100).

The changes in proteins at high pressure have been attributed to the pressure induced unfolding of the protein chains, affecting the tertiary structure: high pressure only affect non-covalent chemical bonds, leaving covalent bonds intact (primary structure). Hydrogen bonds, which stabilize the α-helix and the β-sheets don't seem to be significantly influenced by pressure treatment (93, 99, 100). This effect is important since it is of interest to inactivate or change the catalytic ability of certain enzymes, and generally it occurs above 200 MPa (99, 101, 102).

Pressure can also inhibit the availability of energy to microorganisms by affecting energy-producing enzymatic reactions. The effect of high pressure processing varies between different enzymes and reflects the structure of each enzyme (93, 99).

The destruction of microorganisms is related, but not limited, to the effect on proteins, since the fluidity and permeabilization of cell membranes plays an important role in the viability of the bacterial cells. With pressure they became rapidly impermeable to water and other compounds and occurs a decrease in the functionality of the protein-lipid interactions. In-general, Gram-positive bacteria are more resistant to the high pressure processing than Gram-negative bacteria, however, the extent of inactivation depends on several parameters such as the type of microorganism, the pressure level, the temperature, time, pH and composition of the food or the dispersion medium (101, 103).

The cell membrane is generally acknowledged as the primary site of pressure damage in microorganisms. It was demonstrated that the cell suffers physical damage when it was noted that the cells leaked ATP and UV-absorbing material, and increased

the uptake of fluorescent dyes that don't usually penetrate the membranes of healthy cells (104-108).

In the case of DNA, pressure also affect de DNA structure and function, causing more stabilization of the hydrogen bonds and stacking interactions. This fact leads to an increasing of the duplex to single-strand transition temperature, indispensable for replication, translation and transcription (109, 110).

The effect of high pressure in ribosomes gives the impression to be the main factor of cell death. This inactivation of the ribosomes is caused by a dissociation of the two ribosomal subunits (107, 111).

Cells in different life cycles behave differently when exposed to high pressure treatment. A cell in the exponential phase is more susceptible to high pressure treatment than a cell in the stationary phase. This effect is related to changes in the membrane during the exponential phase, such as loss of osmotic responsiveness and loss of proteins and RNA to the extracellular medium (100). However the cell cycles change with the pressure, because the cell division is also indirectly influenced by variations of structure and functions of the remains cellular organelles (112).

Cell morphology also suffers from the effects of high pressure. These include nodes and bud scars on the cell surface. Another reported effect was the formation of cavities between the cell membrane and the cell wall (112).

In terms of Figures, a substancial count reduction, above four logarithmic units, in the number of vegetative of microorganisms is achieved when a pressure treatment of 400-600 MPa is exerted at room temperature (100, 101).

Since a pressure treatment will not completely inactivate all microorganisms, but rather injure part of the population. These injured cells are able to recover if the medium conditions are favorable and can, consequently, influence products (100).

Bacterial endospores are more resistant to the high hydrostatic pressure treatment than vegetative bacteria, some are even able to withstand pressures of over 1000 MPa. The spores from *Clostridium botulinum* are among the most pressure resistant. However, relatively lower pressures (200MPa) can induce germination. This suggested the use of pressure cycles to inactivate spores: the first cycle intends to promote the germination and the second cycle intends on (93, 100, 103).

To achieve acceptable inactivation of spores it is necessary to combine high pressure treatment with other methods, with thermal treatment being the most studied method of inactivation. This method makes use of the property of adiabatic heating, in

which a product is heated to approximately 100ºC, and then compressed, increasing the temperature by 3-9ºC/100 MPa. The temperature automatically lowers when the product is decompressed (103).

The impact of pressure on organisms depends of the duration and extant of the treatment and other environmental parameters. The chemical composition of the substrate during treatment may influence significantly the response of microorganisms to pressure. Molecules like proteins, carbohydrates and lipids can exert a protective effect, therefore it is necessary a study of the characteristics of each product to access the effect of pressure in the microorganisms. In example, it was verified that inactivation results of *E.colli* in laboratory conditions were much more effective than the ones verified in poultry meat and milk. Cations such as Ca^{2+} can be baroprotective, explaining the results verified in milk, as well as other foods (100, 113).

Experiments with water activity indicated that in a product with low water activity it is harder to inactivate microorganisms. pH has a different effect compared with water activity: lower pH can act synergistically with high pressure processing to inactivate microorganisms. As the pH is lower, cells are more susceptible to pressure inactivation, and sublethally injured cells fail to repair and die more rapidly (100, 113).

1.2.2 Applications

The high pressure has applications in different scientific domains and was strongly dependent on the development of the related technologies. Firstly, high pressure technologies are mainly applied in Physics and Chemistry, in studies of the compressibility of gases and liquids (114-116). The research in these areas proceeds, with successful milestones such as the synthesis of diamond (117, 118) and the production of the NH_3, that was the first industrial application of this technology (119).

Different processes were then developed in Materials Science, such as, polymerization (120), hydrothermal crystal growth of materials (121), synthesis and crystal growth of diamond for the development of machining or cutting processes for super-hard alloys (122), elaboration of CrO_2 as fine particles well de fined in size and morphology for magnetic recording applications (123), high pressure sintering of dense ceramics (124).

These first successes and the technological developments associated with high pressure led to an increase of the use of this thermo-dynamical parameter in different

scientific domains such as Physics (116), Chemistry (materials chemistry (125) or organic chemistry (126)), Geosciences (127), and as described below in Biosciences (128).

However, even in the century XIX, was started studies in the effect of high pressure on living systems, with high pressure treatment to kill bacteria such *Escherichia coli* and *Staphylococcus aureus*, in 1895 by Royer (129). A couple of years later Hite start research the conservation if milk under high pressure (130).

In the start of the XX century, Bridgman studies the coagulation of albumen under pressure (131) and Hite, with the goal to preserve fruits and vegetables, develop hydrostatic pressure for the inactivation of some microorganisms (132).

James and Jacques Basset *et al.* (133-138), in the period between 1932 and 1956, they studied the pressure resistance of bacteria and viruses, effects on antigens, antibodies, questions associated to the immunogenicity and the role triggered on tumors, leading to a research on the evaluation of the vaccinal and antigenic properties of bacteria inactivated under high pressure.

1.2.2.1 Food development

In the field of biosciences the research began to center mainly in food applications, where the aim is the elimination of microbial pathogens and extension of the shelf-life, with Timson and Short's investigating the pressure effect in microorganisms of raw milk, Gould and Sale´s in the germination of spores under pressure (139), Wilson's in the sterilization of low-acids foods (140), Elgasim and Kennisck's on beef proteins (141).

During the eighties, research involving high Pressure and food processing had evolved with the works of Morild *et al.* (1981) (142) on the high pressure effects on enzymes, Heremans *et al.* (1982) (143) on the high pressure effects on proteins and other biomolecules, Popper and Knorr (1990) (144) on the applications of high pressure homogenization for food preservation, Hoover *et al.* (1989) (145) on the biological effects of high hydrostatic pressure on food micro-organisms, Farr at al. (1990) (146) on the high pressure technology in food industry.

Strong efforts for setting up new food processes were conducted in Japan in particular. Such developments in Japan can be explained by different factors: the difficulty to use ionizing treatments, the preservation of the organoleptic properties of

the raw material in agreement with the Japanese cooking culture, and the development of original processes supported by a specific technique (high pressure) (147-150).

Such research activity led to: a strong interest, in basic research, to explain the mechanisms of microorganisms inactivation under high pressure, and new industrial processes for food preservation (the first food product stabilized under high pressure reached the Japanese market in 1993) (99, 151-156).

Microorganisms can be inactivated without affecting food molecules that contribute to the flavor or texture of the food product, thus increasing the shelf life and safety of food products. Also, since high pressure processing can be done at mild or chill temperatures, there is little damage to heat sensitive nutrients or natural flavours and colours, maintaining the quality of the processed food (93, 104).

Over the last fifteen years high pressure technology in food processing has steadily increased.

Several products are now available on the market in different countries: fruit juices, jam, tofu, ham, shellfish, and biopolymers (such as proteins or starches).

General papers dealing with the development of pressure indicators for HHP processing of foods, food safety or "commercial opportunities and research challenges in high pressure processing of foods" underline the strong interest of such non-thermal treatment. Products like fruit jams and sauces first became available in Japan in the early 1990s. Treatment of fruit jams with around 400MPa for up to 5 min at room temperature can significantly reduce the number of microorganisms. Despite the effects of browning and flavor changes caused by enzymatic activities these products were able to maintain stability for 30 days with superior sensory quality compared to products conventionally treated (100).

For fresh fruit juices, a processing at 400 MPa for a few minutes at mild temperature enables the product to maintain its stability for 30 days, eliminating yeasts and moulds, as well as pathogens. In Portugal, a line of pressure treated fruit juices and prepared foods became commercially available in 2007 by the company Sonatural (100, 157).

Despite difficulties found in the processing and conservation of pressure treated vegetables, due to high pH along with the possibility of surviving spores, there is a product whose market sales have been steadily increasing: guacamole. The increase in sales is attributed to the superior sensorial quality of pressure treated guacamole in comparison with heat treated or frozen product. The treatment uses 500 MPa of pressure

during 2 minutes, and the shelf-life is extended from 7 to 30 days in refrigerated conditions. This process as already been approved by the U.S. FDA (100, 113).

A product that was initially available in Spain (and later in the US), is vacuum packed sliced cooked ham, along with other delicatessen meat products. These are treated in flexible pouchs for a few minutes at 500 MPa. All the sensory properties remain unchanged and shelf-life is extended to 60 days in chilled storage. This kind of products have the risk of contamination of microorganisms like *L. monocytogene,* a know pathogen (100).

The last example of a product treated with hydrostatic pressure processing belongs to oysters, which are usually eaten raw or lightly cooked. After an initial treatment with high pressure, in order to inactivate *Vibrio* spp., it was discovered a beneficial effect: the aductor muscle is released from the shell, making the oysters easy to open. The oysters became commercially much more attractive and are sold with virtually no changes in the sensory quality. The typical treatment consists in subjecting the product to 250-350 MPa for 1-3min. With this product, the treatment of seafoods became a much more interesting business (100, 113).

1.2.2.2 Biotechnology applications

In parallel, marine medium has been used as a model in different cases for the study of microorganisms.

Piezophiles are microorganisms that require pressure above the atmosfepheric pressure for optimal grow rate and consequently they have a inhibitory pressure higher than surface microorganisms. Their discovery has prompted researchers to investigate the survival strategies developed by these microorganisms for their adaptation to high pressure environments (associated in some cases with high temperature). Such a scientific domain has led to works on the adaptation, according to the pressure value, of different components of the living systems such as lipids and biological membranes or proteins (107, 110, 112).

In parallel, over these last years, HHP has been investigated for biotechnological applications.

For example, proteins and enzymes isolated from extremophiles, in particular piezophile and thermophile microorganisms, open the way to new applications in different domains: very sensitive to pressure and temperature parameters in clinical, environmental and food analysis, the construction of nanosensors (158, 159).

The enzyme activity, like temperature, can be controlled by pressure. The functionality and stability of most enzymes are not substantially changed by pressures up to 200 MPa. Before these pressures the activity of some enzymes may decrease or increase. Commonly, if the catalytic reaction is induced by a negative change of volume, then the high pressure will increase the enzymatic activity (160, 161).

Contrary to the use of common perturbing agents, such as temperature, urea, and guanidine that cause drastic modifications in protein structures, high pressure only affects non-polar interactions that are essential in protein folding if this leads to a decrease in volume. Consequently, high pressure denaturation of proteins mainly depends on their tertiary and quaternary structures. These possibilities make possible studies of proteins in determinate intermediate conformations, demonstrating the presence of many partially folded conformations between the completely unfolded and fully folded states, with in particular the aggregation and amyloidogenises. The interest of this lies for example in the fact that some cancers have been found related to protein misfoldings (128, 162-172).

Most problems of allergenicity and digestibility are related to proteins. To reduce these problems the most common solution for hypoallergenic products is the enzymatic digestion with proteases. When the treatment is applied under high pressures, the proteins may become more accessible by proteases, resulting in a product with lower allergenicity (160, 172-175).

Other application lies in a genetic transformation of cells, which is a common procedure in bioengineering development, but is often limited by the efficiency of transformation as only few cells take up the plasmid of interest. It was demonstrated that high pressure treated plasmids (pUC18 and pBR322) exposed to high pressure treatment (200 and 400 MPa respectively) present an increased capacity to transform competent cells. This observation can be explained by the stabilization of hydrogen bonds under high pressure conditions, as many properties of the plasmids, such as their mobility and their ethidium bromide binding efficiency, are modified after high pressure treatment (176).

With high pressure technology, a new method of cell extraction has been developed by Pressure Biosciences Inc., using the combination of pressure cycling technology (PCT) and extraction solvents that allow dissolution and partition of each type of molecules into separate fractions. The use of this technology followed by centrifugation leads to good recovery of proteins and lipids, high yields of intact DNA or RNA,

without any further purification steps. It is also possible to adjust the PCT conditions to recover intact organelles such as mitochondria (177-179).

In the case of the cryopreservation, after high pressure treatment, mammalian cells appear to be more resistant to this process (172). For example, fresh bull semen treated under high pressure (40 MPa for 90–120 min) followed by freezing shows more viability, motility and fertility than semen directly freezed. Post-thaw survival of frozen mouse and bovine blastocysts, and pig oocysts, is also enhanced after high pressure treatment. It is suggested that this increase of freezing resistance may be due to the production of "shock proteins" (180-183).

By other side, it is often hypothesized that pressure-inactivated pathogens would be able to stimulate the immune system and thus could be used as a vaccine. Indeed, the unfolding of proteins under high pressure unmasks antigenic sites and may increase immunogenic properties of pressure-treated proteins, killed viruses and micro-organisms (160, 161).

In addition to biocatalysis, the use of HP also shows promise in microbial fermentations, where few work was done. For example, it was recently shown that at 10 MPa the fermentation of glucose to ethanol in *Saccharomyces cerevisiae* proceeded three times faster and gave a slightly increased yield when compared with the same fermentation at ambient pressure (Fig. 8) (184).

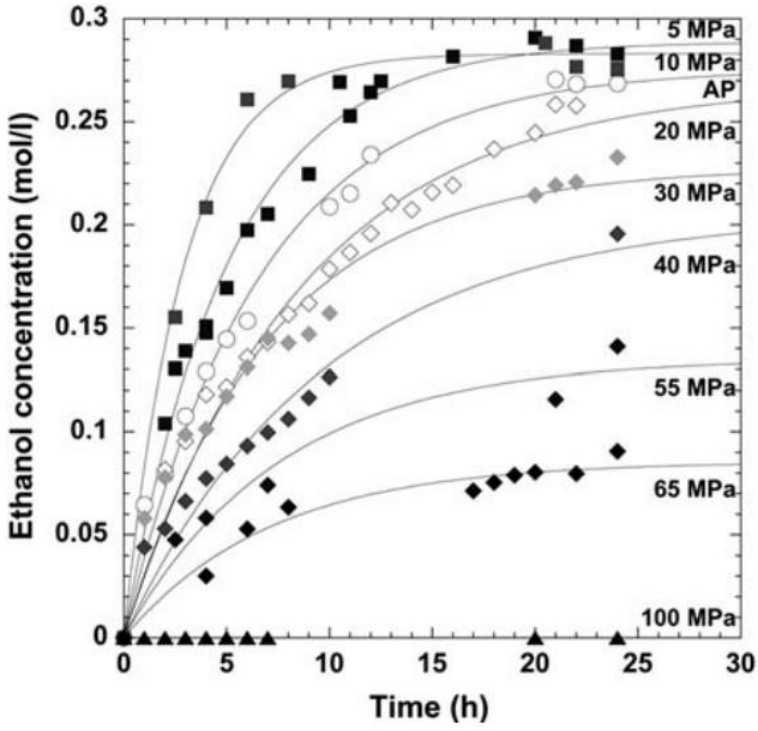


Figure 8 – Kinetics of ethanol production by *Saccharomyces cerevisiae* as a function of pressure to 100 MPa (184).

Furthermore, it was found for *Clostridium thermocellum* that application of pressure of 17 MPa dramatically redirected the fermentation products of cellobiose from organic acids, such as acetate, at atmospheric pressure to ethanol at higher pressure, leading to a 60-fold increase in the ratio between ethanol and acetate. This effect could be undoubtedly exploited for enhancing the industrial production of bioethanol by microbial fermentation (185).

1.2.3 Equipment

The construction of high pressure machinery is a specialized and expensive operation. However, since there are needs for high pressure treatments in industries other than the food industry there is already a solid background in the construction of high pressure processing equipments.

The mains components of a high pressure system are: a pressure vessel and its closure, a pressure generation system, a temperature controlling device and a material handling system (186).

The pressure vessel is usually made from a high tensile steel alloy monobloc, meaning that it is forged from a single piece of material. When higher pressures are used, the vessel changes, and pre-stressed or wire-wound vessels are used.

In operation, after removing all air, a pressure transmitting medium, either water or oil, is pumped from a reservoir into the pressure vessel using a pressure intensifier until the desired pressure is reached. This is indirect compression. Direct compression is generated by pressurizing a fluid by a piston, driven at its larger diameter end by a low pressure pump (Fig. 9) (93, 186).

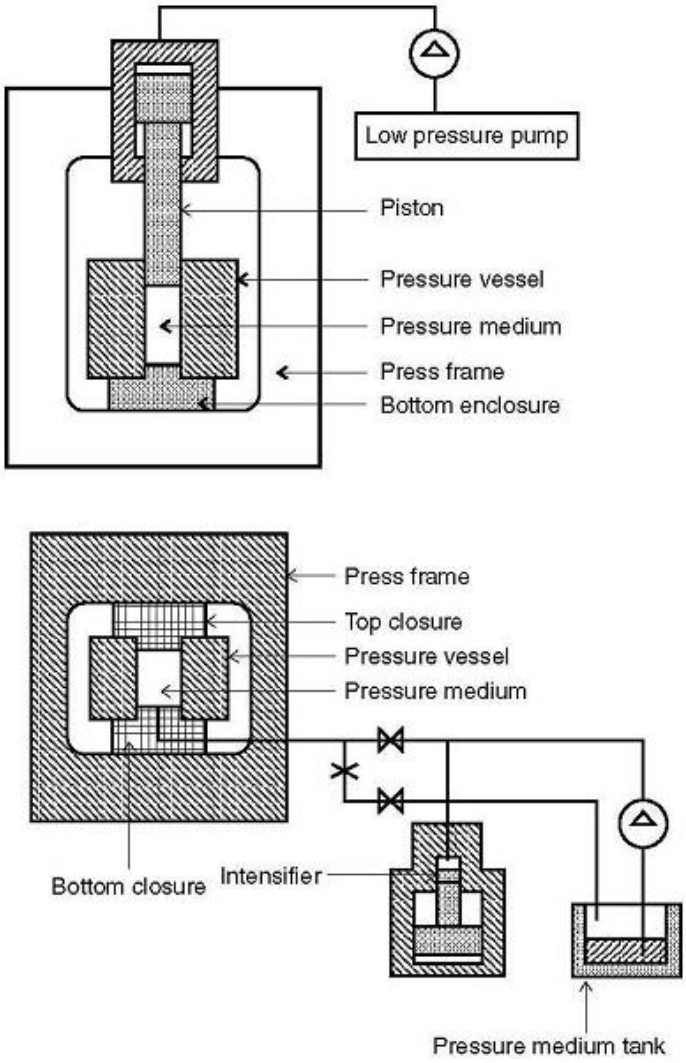


Figure 9 – Scheme of direct and indirect compression equipment for high pressure processing (93)

There are two methods of processing foods in high-pressure vessels: in-container processing and bulk processing. Because foods reduce in volume at the very high pressures used in processing, water has a 15% volume reduction at 600 MPa, there is considerable stress and distortion to the package. For the time being, conventional plastic and foil pouches are suitable, and research is continuing for the optimum package. Bulk handling is simpler, requiring only pumps, pipes and valves, however it is only suited for pumpable foods (186).

1.3 High pressure and enological sector

In the Enological sector some studies involved HP treatments have been made. The main objective of studies done using HP treatments in wine is the microorganisms' inactivation. The aims of these treatments are preserve grape juice and must before the fermentation and pasteurize wines after the fermentation to reduce the levels sulfur dioxide (SO_2) needed to store the wine (187-190).

The SO_2 is probably one of the most versatile and efficient additives used in winemaking due to its antiseptic and antioxidant properties, for minimizing phenolic polymerization rate and color loss during aging. Some SO_2-derived compounds, namely sulfites, may cause allergies problems (191-195).

The first report about HHP on wine was from Lonvaud-Funel *et al.*, in 1994 (196). They treated Sauternes, a sweet white wine from Bordeaux (France), with HHP at 200-400 MPa, for various holding times. The initial yeast count of 6.88 $\log_{10}$ was decreased to 4.88 and 4.54 $\log_{10}$ after a HP treatment at 200 and 250 MPa, respectively. Pressurization at 370 and 400 MPa for 5 min, 300 MPa for 10 and 20 min, 350 and 400 MPa for 15 min resulted in complete elimination of yeasts.

The second study was done by Delfini *et al.* (48) with microorganisms, such *Leuconostoc oenos, Lactobacillus spp.*, *Acetobacter,* and *Botrytis cinerea*, added to Moscato wines obtained from Barbera grape must. The HP treatments used range 300-600 MPa, at 20°C, demonstrate a strong antimicrobial effect with no variation of the color parameters compared with untreated wine.

In the next years Tonello *et al.* publish 3 articles (197-199) relative of this matter. In the first was reported the use of HP to inactivate yeasts, lactic and acetic acid bacteria in different wines (white, rosé and red). The tests are performed with 300 and 400 MPa, at room temperature and various holding times. It was achieved a complete elimination of yeasts, lactic and acetic acid bacteria. Other important conclusion is that the ethanol addition accelerates inactivation of yeasts, whereas sugar addiction had no effect. In the other two experiences were tested wines contaminated with different strains of *S. cerevisiae, S. ludwigii*, *Pediococcus* and *A. aceti*. They verify that acetic acid bacteia was pressure resistant than lactic acid bacteria and yeasts.

In the case of grape musts, around 1997, Castellari *et al.* (200) demonstrate at around 900 MPa the activity of grape musts' polyphenoloxidase (PPO) enzymes was

clearly lowered (up to 16%). However at lower pressures, Del Pozo-Insfran *et al.* (201) proves that PPO enzymes activity may be increase depending of time and pressure.

Puig *et al.* (188) also investigated the microbial and biochemical stabilization of wines by use HP processing. They tested two yeasts (*Saccharomyces cerevisiae* and *Brettanomyces bruxellenis*), two lactic acid bacteria (*Lactobacillus plantarum* and *Oenococcus onei*) and two acetic acid bacteria (*Acetobacter aceti* and *Acetobacter pasteurianus*) in white and red wine. The treatments were performed using a pressure of 400 or 500 MPa, for 5 or 15 min, with 4 or 20 °C. They reached a complete inactivation of yeasts, lactic and acetic acid bacteria, with no baroprotective effect of sugar addiction on yeasts and bacteria. They also conclude there is no effect of HP treatment of PPO activity, alcohol level, total and volatile acidity, free and total SO2, protein stability, malic acid, lactic acid, reducing sugars and pH. The organoleptic characteristics in treated wine remain equal of non-treated.

In 2001 Daoudi *et al.* (187) studies the effect of 400 MPas treatment on the colour and sensory characteristics of white grape juice, during storage at 4 °C for 60 days. These characteristics of pressure-treated sample remained more stable than those of the control juice.

Around 2006, Mok *et al.* (190), show the effect of pressure treatments ranging from 100 to 350 MPa, up to 30 min, at 25°C on aerobic bacteria, yeast, and lactic acid bacteria of wine. This research group demonstrates that the microbial inactivation increased with the pressure and time, the aerobic bacteria were more susceptible to the HP treatments than yeasts and lactic acid bacteria, and there is no changes detected in the aroma, taste, mouth-feel and overall sensory quality between the HP treated and untrated samples.

Corrales *et al.* (202) revealed that a combined temperature and pressure treatments (600 MPa and 70°C) during 1 h produce a decrease of anthocyanins, while in a treatment of 10 min, no differences in anthocyanin composition or antioxidant activity was observed.

The main disadvantages of HPP treatments for preserve wine is the current impossibility to be used as a continuous process and the needed of new bottle packing more resistant and flexible, because the HP treatment is applied after the bottling. For this limitation raze a new challenge of product presentation.

1.4 Objectives:

The malolactic fermentation is a process with high interest for the wine industries, characterized by its long duration. At this moment, with the industrial equipment production growth, high pressure promise to be a technology to improve this process with low environmental impact.

Taking into account the pathway to move toward more sustainable industries, particularly wine industries, it is necessary to develop processes and production which save costs and are more profitable. So the aim of this work is optimize the malolactic fermentation duration, never compromising the environmental impact of production.

The work developed in the present thesis has its main focus in the study of the effect of the high pressure treatment in malolactic fermentation of *Oenococcus oeni*. Additionally, it was also studied the effect of this treatment on sugar metabolism and microbial growth of this specie. To achieve these goals, *Oenococcus oeni* was inoculated in a modified MRS medium (with L-malic acid) and then pressurized at different pressures and holding times. After the treatment the samples were incubated at atmospheric pressure. The following analyses were conducted:

- L-malic acid and L-lactic acid concentration analysis for malolactic fermentation (L-malic acid decarboxylation);
- Glucose and D-lactic acid concentration analysis for sugar metabolism;
- Optical density at 660 nm for microbial growth.

CHAPTER II

MATERIALS AND METHODS

2.1 Liquid growth medium

To study the effect of high pressure in the growth and metabolism of the *Oenococcus oeni* it was necessary to select a strain and an appropriate liquid growth medium.

The liquid medium used was a modified MRS broth. The MRS broth is a non-selective medium based on the commercial formulations of de Man, Rogosa and Sharpe (MRS). Developed in 1960, this medium was designed to favour the luxuriant growth of mainly *Lactobacilli* for laboratorial studies. It contains sodium acetate, which suppresses the growth of many competing bacteria. To obtain an optimal growth medium for *Oenococcus oeni*, it was added L-malic acid and ethanol to MRS Broth

The modified MRS broth enabled the study of the L-malic acid degradation metabolism, the sugar metabolism and the microbial growth.

This modified MRS broth was composed by 54.3 g/L of commercial MRS broth (Liofilchem Diagnostici), 1.0 mL/L of tween 80 (according the commercial MRS broth protocol), 3.5 g/L of L-malic acid (Sigma, >95%) and 5 % (v/v) of ethanol (Riedel-de Haën, >99.8%). All the components were dissolved in distilled water and the final pH was 5.0, adjusted with HCl solution. The medium was sterilized at 121.1 °C, during 15 min. The ethanol was only added after the sterilization and under aseptic conditions.

Table 1 – Commercial MRS broth composition

Peptone	10.0 g/L
Beef extract	10.0 g/L
Yeast extract	5.0 g/L
Glucose	20.0 g/L
Triammonium citrate	2.0 g/L
Sodium acetate	5.0 g/L
Magnesium sulphate	0.2 g/L
Manganese sulphate	0.05 g/L
Di-potassium phosphate	2.0 g/L

2.2 *Oenococcus oeni* and inoculation

The strain used was MBR Lalvin VP 41 ® (Lallemand), which was isolated in the Abruzzi region of Italy during an extensive European Union collaboration to research natural *Oenococcus oeni* strains. Lalvin VP 41's positive enhanced mouthfeel contribution stood out in comparison with other malolactic bacteria strains during tasting. The very good implantation, high alcohol and SO_2 tolerance of this strain make it a reliable malolactic fermentation culture to use when a significant impact on wine structure is desired, especially when using security yeasts for primary fermentation that are producing high levels of total SO_2.

The concept MBR ® is a process of acclimatization, specifically developed by Lallemand, where the bacteria are subjected to chemical and biophysical conditions of stress, thereby increasing its resistance. Packed bacteria are robust and have the ability to conduct malolactic fermentation in a safe, even in difficult conditions. Bacteria are directly applicable.

The inoculation was done according to the seller protocol. In the first step, called rehydratation, the bacteria was suspended in distilled water in a concentration of 50 g/L, at, during 15 min. Then, for the inoculation, this suspension was transferred to modified MRS broth, at 15-25 °C, in a concentration of 0.2 mL/L. Several Polyamide/Polyethylene 90 My bags, previously UV treated, were filled with 5 mL of inoculated medium and sealed. The bags dimension was 5 cm x 2.5 cm. All this procedures were executed at aseptic conditions, in a laminar flux chamber.

2.3 Pressure treatments

The pressure treatments were carried out using a hydrostatic press from Unipress Equipment, Model U33 (Warsaw, Poland), with a pressure vessel of 100 mL (35 mm diameter and 100 mm height), surrounded by an external jacket, connected to a thermostatic bath to control the temperature. The unit has a maximum working pressure of 700 MPa and a working temperature between -20 ºC and 100 ºC. The pressure-transmitting fluid was a mixture of propylene glycol and water (40:60). All treatments were performed at time following inoculation.

. The temperature of the pressure vessel was set to 24 ºC. For each pressure experiment it was necessary to proceed to a correspondent control assay, from the same inoculated medium.

After the pressure processing, the treated bag samples were incubated in an oven, at atmospheric pressure and 24°C, with the control bag samples. During the fermentation time several control and pressurized samples were removed and stored -80°C.

The treatments executed were 50 MPa during 8 h; 100 MPa during 0.5, 8 and 60 h; and 300 MPa during 0.5 h. The holding time didn't exceed the 60 h, derived from security questions of the pressure equipment.

No more treatments were done, because in the initial phase of this work some experiments were performed without success, even in the control samples the fermentation did not occur. Taking in account the experimental time (around 13 days) for each experiment and the analysis time, these failed experiments accounted for 1.5 months of the total work time. The reason for the inadequate results was an improper conservation of the bacterial lot. After that, a new lot was purchased and maintained under adequate conditions, leading to successful fermentations.

2.4 Microbiology and optical density

The microbial growth was measured at 660 nm. The measurements were executed in 96-microwell plate (*Brand*, *polystyrene F96*) and analyzed in a Thermo Scientific Multiskan GO spectrophotometer. The volume used was 300 µL. The microbial growth measurements were calibrated against empty wells.

Plate count microbiology was also evaluated, in order to relate the optic density at 660 nm with the colony-formers units (CFU). The prepared medium was the same that was used in the fermentation studies, but with agar to become solid. Likewise, the medium is composed of 68.2 g/L of commercial dryed MRS Agar (tween 80 (1.0 g/L) and agar-agar (14.0 g/L) (*Merck*) are include in commercial medium) and 3.5 g/L of L-malic acid (*Sigma, >95%*). The final pH was 5.0, adjusted with HCl solution.

The medium components were dissolved in distilled water and sterilized with Ringer solution (Merck) and all accessories needed, at 121.1 °C during 15 min.

The inoculation was performed in a laminar flow chamber, by pour-plate method in commercial sterilized Petri dishes. Several dilutions were made with Ringer solution.

The Petri dishes were incubated at 27°C during 12 days.

2.5 Glucose quantification

The determination of the concentration of reducing sugars was made using the 3,5-dinitrosalicylic acid (DNS). The quantification of the reducing sugars is based on the reduction of DNS in alkaline solution. The carbonyl groups of the sugar is oxidized to carboxyl, resulting in aldonic acids, while the DNS became 3-amine-5-nitrossalicylic. This compound can be measured by colorimetry at 540 nm.

To prepare the DNS alkaline solution, 10 g of DNS were weighted and dissolved in 200 mL of a 2N NaOH solution. The solution was then heated and stirred intensively. Simultaneously, a solution of 300 g of potassium tartrate in 500 mL of distilled water was prepared and heated (with intense stirring). Both solutions were mixed and stirred. Distilled water was added to make up 1 L.

To determine the reducing sugars concentration, 1.0 mL of sample (previously diluted) was added to 1.0 mL of DNS alkaline solution in a glass tube. The tube was vortex stirred and heat at 100°C during 5 min. The reaction was then stopped in ice during a few minutes and 10.0 mL of distilled water were added to the reaction medium. Finally the tube was vortex stirred and the absorbance measured at 540 nm. In the absorbance analysis a 96-microwell plate was used (Brand, polystyrene F96) with 300 µL of sample and analysed in a Thermo Scientific Multiskan GO spectrophotometer. A standard curve was determined in advance.

2.6 Analytical test kits

Analytical test kits were used to measure the concentration of L-malic acid, L-lactic acid and D-lactic acid. All the kits were purchased from *NZYTech, Lda. - Genes and Enzymes*, and stored at 4°C. The analytical test kits are customized for 1 cm cuvettes, but the protocol was adapted and performed in a 96-microwell plate (Brand, polystyrene F96) and analyzed in Thermo Scientific Multiskan GO spectrophotometer. For each analytical test kit it was calculated a calibration curve, constructed with diluted standards, and all samples were correctly diluted according to the concentration range.

2.6.1 L-malic acid

The L-malic acid analytical test kit was based in the action of two enzymes, L-malate dehydrogenase (L-MDH) and Aspartate aminotransferase (AST), leading to the stoichiometrical formation of NADH from L-malic acid (Eq. 5). The concentration of NADH was measured at 340 nm.

$$L - malate + NAD^+ \xrightarrow{L-MDH} Oxaloacetate + NADH + H^+$$

$$Oxaloacetate + L - glutamate \xrightarrow{AST} L - aspartate + 2 - oxoglutarate$$

Equation 5

The purpose of the Aspartate aminotransferase (AST) is the oxaloacetate consumption in order to make a complete conversion of the L-malic acid to NADH, by the L-malate dehydrogenase (L-MDH).

In each microwell it was added 192 µL of water, 10 µL of solution 1 (glycylglycine buffer (1 M, pH 10.0) plus L -glutamate (1 M) and sodium azide (0.02% w/v) as a preservative), 10 µL of solution 2 (NAD^+ (63.33 g/L) plus polyvinylpyrrolidone (10g/L)), 2 µL suspension 3 (Aspartate aminotransferase (EC 2.6.1.1) suspension (600 U/mL)) and 10 µL of sample. Then the plate was incubated and shaken at 25°C, during 3 min, and measured the absorbances. After that it was added 10 µL of suspension 4 (L-Malate dehydrogenase (EC 1.1.1.37) suspension (3,000 U/mL)) and the plate was shaken and incubated at 25°C, during 3 min and the absorbances measured with 5 min interval until the end of the reaction.

2.6.2 D-lactic acid

The D-lactic acid analytical test kit was centered in the activity of two enzymes, D-lactate dehydrogenase (D-LDH) and, leading to the stoichiometrical formation of NADH, from D-lactic acid (Eq. 6). The concentration of NADH was measured at 340nm.

$$D - lactate + NAD^+ \xrightarrow{D-LDH} Pyruvate + NADH + H^+$$

$$Pyruvate + D - glutamate \xrightarrow{D-ALT} D - alanine + 2 - oxoglutarate$$

Equation 6

The goal of the D-alanine aminotransferase (D-ALT) is the pyruvate intake to make a complete conversion of the D-lactic acid to NADH, by the D-lactate dehydrogenase (D-LDH).

In each microwell it was added 142 µL of water, 50 µL of solution 1 (Glycylglycine buffer (0.5 M, pH 10.0), D-glutamate (0.5 M) and sodium azide (0.02% w/v) as a preservative), 10 µL of solution 2 (NAD^+ (69.09 g/L) plus polyvinylpyrrolidone (10.09 g/L)), 2 µL suspension 3 (D-Alanine aminotransferase (D-ALT, 1300 U/mL) in ammonium sulphate (3.2 M)) and 10 µL of sample. Then the plate was incubated and shaken at 25°C, during 3 min, and measured the absorbances. After that it was added 10 µL of suspension 4 (D-Lactate dehydrogenase (D-LDH, 400 U/mL) in ammonium sulphate (3.2 M)) and the plate was shaken and incubated at 25°C, during 5 min and the absorbances measured with 5 min interval until the end of the reaction.

2.6.3 L-lactic acid

The L-lactic acid analytical test kit was focused in the action of two enzymes, L-lactate dehydrogenase (L-LDH) and D-alanine aminotransferase (D-ALT), leading to the stoichiometrical formation of NADH, from L-lactic acid (Eq. 8). The concentration of NADH was measured at 340nm.

$$L - lactate + NAD^+ \xrightarrow{L-LDH} Pyruvate + NADH + H^+$$
$$Pyruvate + D - glutamate \xrightarrow{D-ALT} D - alanine + 2 - oxoglutarate$$

Equation 7

The aim of the D-alanine aminotransferase (D-ALT) is the pyruvate degradation to make a complete conversion of the L-lactic acid to NADH, by the L-lactate dehydrogenase (L-LDH).

In each microwell it was added 142 µL of water, 50 µL of solution 1 (Glycylglycine buffer (0.5 M, pH 10.0), D-glutamate (0.5 M) and sodium azide (0.02% w/v) as a preservative), 10 µL of solution 2 (NAD^+ (69.09 g/L) plus polyvinylpyrrolidone (10.09 g/L)), 2 µL suspension 3 (D-Alanine aminotransferase (D-ALT, 1300 U/mL) in ammonium sulphate (3.2 M)) and 10 µL of sample. Then the plate was incubated and shaken at 25°C, during 3 min, and measured the absorbances.

After that it was added 10 µL of suspension 4 (L-Lactate dehydrogenase (L-LDH, 400 U/mL) in ammonium sulphate (3.2 M)) and the plate was shaken and incubated at 25°C, during 10 min and the absorbances measured with 5 min interval until the end of the reaction.

CHAPTER III

RESULTS AND DISCUSSION

3.1 *Oenococcus oeni* metabolism

The metabolism of *Oenococcus oeni* was studied in 3 distinct aspects: malolactic fermentation, sugar metabolism and microbial growth.

The malolactic fermentation, as above described, consists in the decarboxylation of the malic acid to lactic acid. In the case of *Oenococcus oeni*, this reaction is catalized by an enzyme, commonly referred as malolactic enzyme. This enzyme, particularly from this bacterial species, catalyzes the conversion of L-malic acid. The products of this reaction are carbon dioxide and only the L-lactic acid stereoisomer. To follow this fermentation, the L-malic acid and the L-lactic acid concentrations were measured.

The typical sugar metabolism of *Oenococcus oeni* is called heterofermentative. The heterofermentative metabolism consists in the production of an ethanol molecule, a carbon dioxide molecule and a lactic acid molecule, from a molecule of glucose. In the case of this bacterial species, only the D-lactic acid stereoisomer is produced from the metabolism sugar. To monitor the sugar metabolism the glucose and the D-lactic acid concentrations were measured.

The Figure 10 resumes the glucose, L-malic acid, L-lactic acid and D-lactic concentrations in fermentation during 296.25 h. The x-axis is correspondent to the period after the inoculation (0 h). In the moment of the inoculation, as expected, the concentration of glucose is around 20.05 g/L and the L-malic acid around 3.61 g/L.

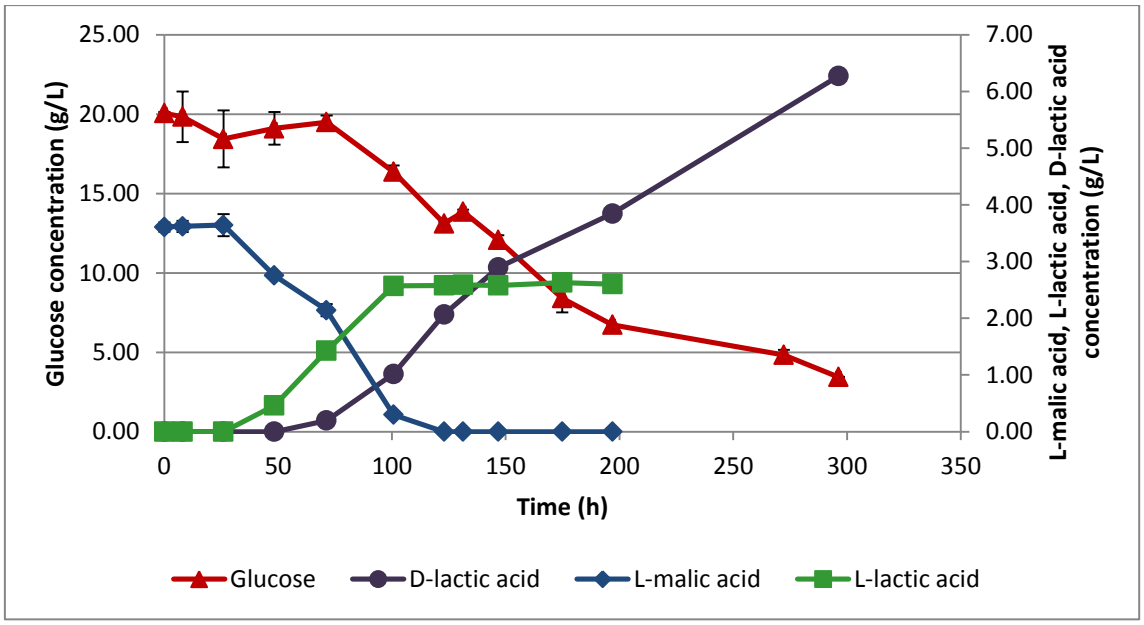


Figure 10 – Concentration of glucose, D-lactic acid, L-malic acid and L-lactic acid during fermentation at atmospheric pressure

In the case of the malolactic fermentation, the L-malic acid consumption starts 25.97 h after inoculation. At 122.97 h the complete consumption of the L-malic acid presented in the medium was reached. Complementarily to the L-malic acid consumption, as expected, L-lactic acid production was verified. Such as the L-malic acid, the L-lactic production starts at 25.97 h and finishes at 100.72 h, reaching 2.60 g/L. At the end of the fermentation, in 1 litre of medium it was consumed 3.61 g of L-malic acid and produced 2.60 g of L-lactic acid, corresponding to $2.69x10^{-2}$ mol and $2.89x10^{-2}$ mol respectively. The molar ratio related to both compounds was 1.00:1.07 (L-malic acid:L-lactic acid) . As expected, this value is very close to 1:1 and the small difference can be attributed to experimental errors.

Saguir *et al.*, while investigating the effect of the essential amino acids in *Oenococcus oeni* metabolism, isolated from an Argentinean red wine, achieved a molar ratio very close to 1:1 (L-malic acid :L-lactic acid) in all media studied. Each medium was deficient in one essential amino acid. The fermentations occurred at pH 4.8 and 30°C (203).

Relatively to the sugar metabolism the initial concentration was 20.05 g/L as expected. After 71.27 h the glucose concentration started to decrease, attaining 3.45 g/L at 296.25 h. The completed glucose consumption was not obtained in the analyzed time. Likewise, the D-lactic acid production began at 71.27 h and at 296.25 h the concentration was 6.27 g/L. During the experimental time it was consumed $9.21x10^{-2}$ mol/L of glucose and produced $6.96x10^{-2}$ mol/L of D-lactic acid. The molar ratio was 1.00:0.76 (glucose:D-lactic acid).

This molar ratio is less than 1:1, because glucose may not be consumed only for product formation, but also for microbial growth (cell division) and cellular maintenance. The cellular maintenance refers to the fraction of substrate consumed by cells to generate the energy required in a number of processes, that do not lead directly to new cell formation or synthesis of extracellular products. These processes include maintaining the concentration gradients, in particular protons and the electric potential across the cell membrane; turnover of the macromolecules, since several categories of macromolecules are very stable, although others (for example enzymes, some cell wall constituents and mRNA) are continuously synthesized and degraded within cells (note that the order of magnitude of the half-life of mRNA molecules is only a few minutes, which allows to control the synthesis of proteins); and cell motility (204).

Rozès *et al.*, studying the effect of phenolic compounds in sugar metabolism of *Oenococcus oeni* CECT 4100, obtained a molar ratio ranging from 1.00:0.89 to 1.00:0.70 (glucose:D-lactic acid) in the presence of different phenolic compounds at 25ºC and pH 4.0 with 6% (v/v) ethanol (205).

Saguir *et al.*, in the previously mentioned work, also obtained a molar ratio ranging from 1.00:0.96 to 1.00:0.72 (glucose:D-lactic acid), by changing the cysteine concentration in the medium (203).

This molar ratio is variable according to the medium and fermentation conditions, since glucose, as described, may be used for different purposes according to the cell necessities, that varies with the fermentation factors.

The Figure 10 clarifies the fact previously presented about malolactic fermentation and glucose metabolism products, because the L-malic acid consumption coincides with the L-lactic acid production, and the glucose consumption with the D-lactic acid production. The molar ratio obtained of L-malic acid against L-lactic acid and glucose against D-lactic acid also elucidates this fact.

The Figure 11 represents the optical density at 660 nm and the colony-formers units during 296.25 h. Both of these analyses reflected the microbial growth during the fermentation, with small differences. The optical density measured the biomass concentration, in other words viable and non-viable cells. On the other hand the colony-formers units count evaluated only viable cells.

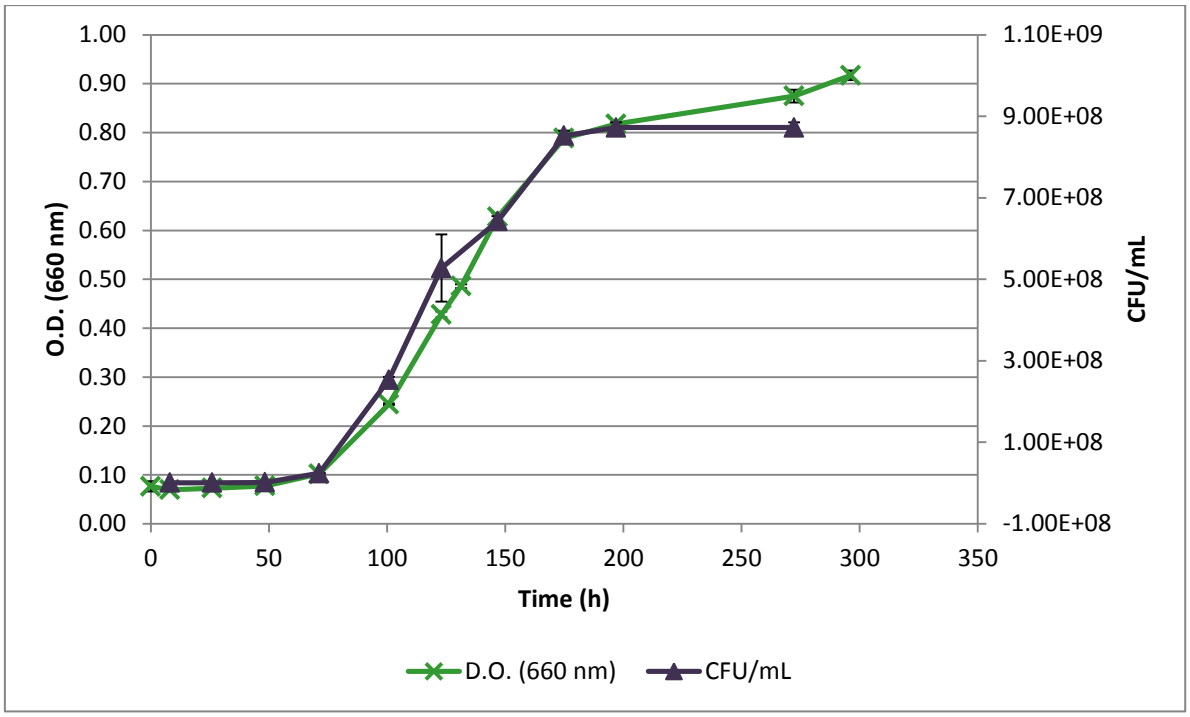


Figure 11 – Optical density (660 nm) and colony-formers units during fermentation at atmospheric pressure

At the moment of the inoculation, the optical density at 660 nm was 0.077 and the colony-formers units was 8.95×10^5 CFU/mL. Both of these values remain stable until 71.27 h. After that, coinciding with the start of glucose consumption and D-lactic acid production, an exponential growth was verified, finishing at 147.85 h with an optical density of 0.788 and colony-formers units of 8.53×10^8 CFU/mL.

The curves of both analysis are very similar (with different units), except at the final of the fermentation. This fact is due to the stationary phase of the microbial growth the cellular doubling rate is equal to the cellular death rate. Therefore it is expected no differences in the colony-formers units and a small growth in the optical density, because the microorganisms keeps doubling increasing the biomass concentration.

The microbial growth, as expected, was closely related with the glucose consumption, because this compound was the main source of energy necessary to this process.

Rozès et al., in the previously mentioned work, obtained with *Oenococcus oeni* CECT 4100 an initial colony-formers units count of about 7×10^7 CFU/mL and at the final of the fermentation about 1×10^8 CFU/mL, in a presence of different phenolic compounds at 25ºC and pH 4.0 with 6% (v/v) ethanol (205).

3.2 50 MPa treatment, during 8 h

The effect of 50 MPa during 8 h after inoculation, in L-malic acid metabolism is represented on Figure 12. The initial L-malic acid concentration was 3.47 g/L ($2.59x10^{-2}$). After 8h pressurization a considerable changes among pressurized and control samples were not verified. In the control samples, a significant L-malic acid degradation started after 24.88 h and stopped before 94.67 h, with total substrate consumption, while the same effect on the pressurized samples only started at 35.02h and finished at 94.67 h.

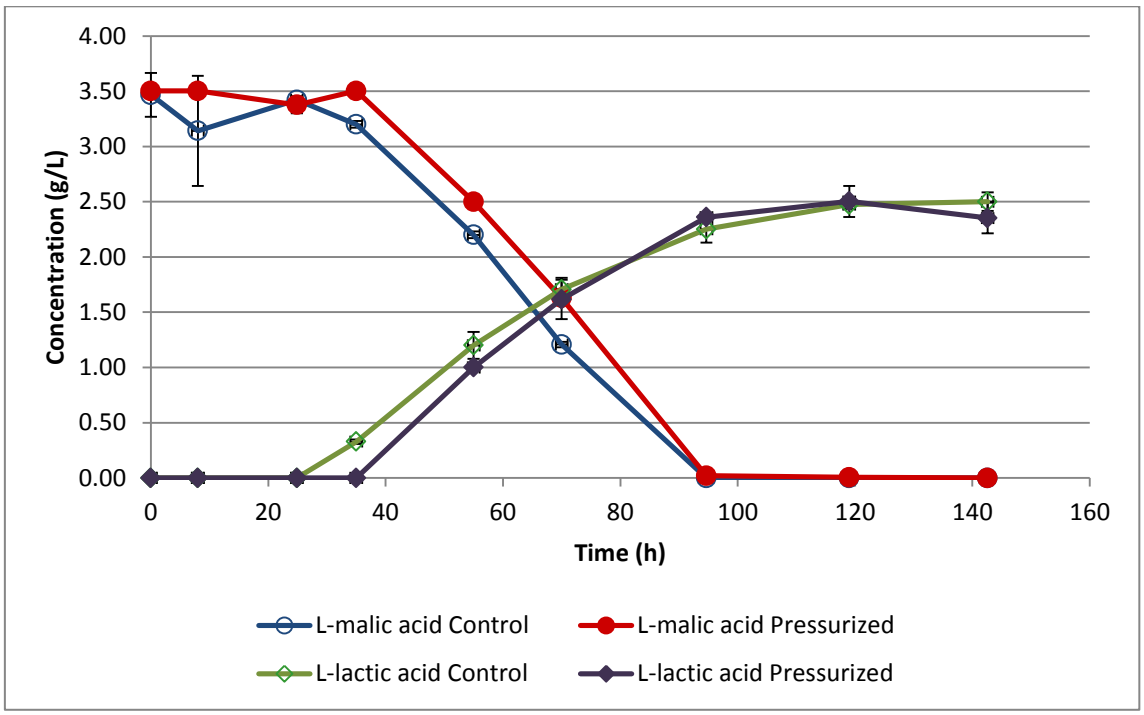


Figure 12 – L-malic acid and L-lactic acid concentration in control and pressurized samples, treated at 50 MPa during 8 h

In the same way of L-malic acid, L-lactic acid production in control samples starts after 24.88 h and terminate before the 94.67 h, reaching a concentration of 2.50 g/L ($2.78x10^{-2}$ mol/L), at 142.58 h of fermentation time, while the same effect on the pressurized samples this production only started at 35.02 h and finished at 94.67 h, with 2.35 g/L ($2.61x10^{-2}$ mol/L) at 142.58 h.

The molar ratio attained for L-malic acid against L-lactic acid was 1.00:1.07 in control samples and 1.00:1.01 (L-malic acid:L-lactic acid) in pressurized samples.

This high pressure processing did not show a substantial effect on the molar ratio, but 10.14 h delay was detected before the start of L-malic acid consumption.

Figure 13 reflects the effect of 50 MPa treatment, during 8 h in glucose consumption and D-lactic production.

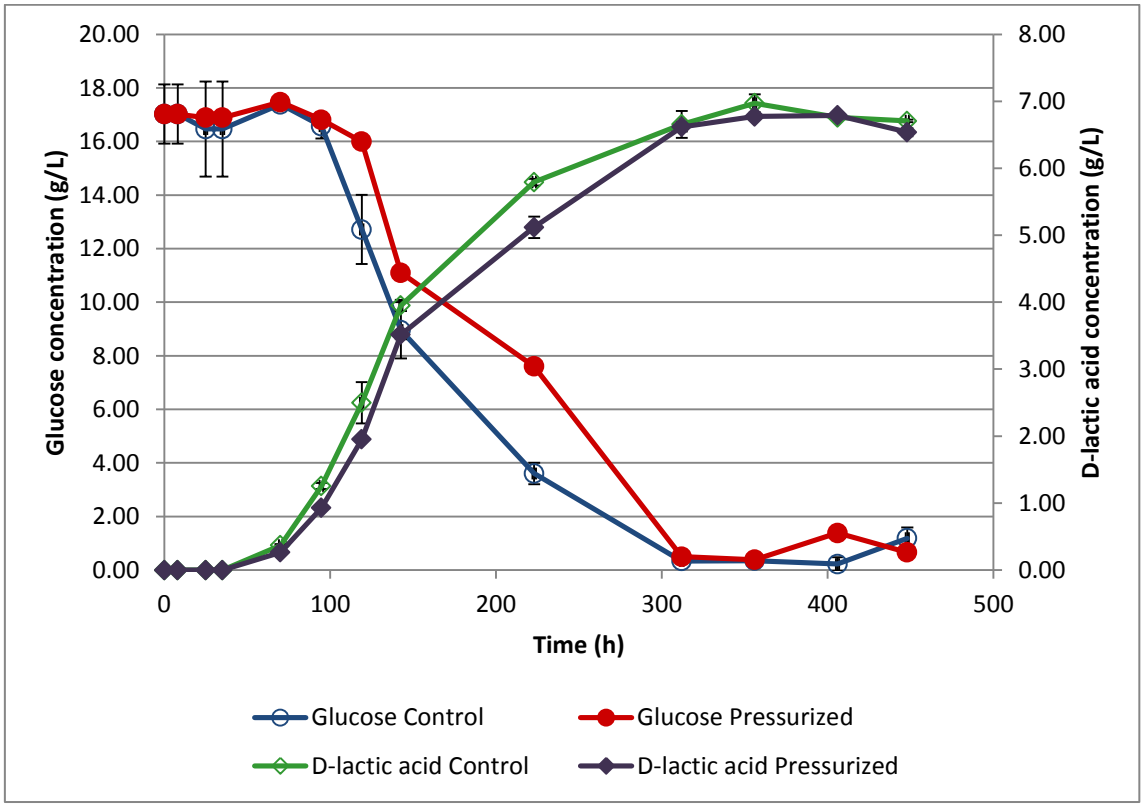


Figure 13 – Glucose and D-lactic acid concentration in control and pressurized samples, treated at 50 MPa during 8 h

The initial concentration of glucose in the medium was 17.02 g/L ($9.45x10^{-2}$ mol/L). In both samples, control and pressurized, the glucose intake begans at 70.03 h and total glucose consumption was detected at 312.12 h.

In the case of the D-lactic acid, the production started at 70.03 h for both samples, reaching a concentration of 6.66 g/L ($7.39x10^{-2}$ mol/L) for control samples and 6.62 g/L ($7.35x10^{-2}$ mol/L) for pressurized samples, at 312.12 h. It was not detected a considerable variation of the D-lactic acid concentration after 312.12 h.

The molar ratio of glucose against D-lactic acid was 1.00:0.78 (glucose:D-lactic acid) in control and pressurized samples.

The glucose intake was simultaneous for both samples, however, the pressure treatment seems to have caused a lower velocity intake of glucose during 24.33 h after the beginning of the consumption of this compound. Therefore the treatment of 50 MPa during 8 h, influences mainly the initial phase of the glucose intake.

The effect of this high pressure treatment in optical density and consequently in microbial growth was represented in the Figure 14. Until the 70.03 h of inoculation, it was not identified a considerable change of this factor for both samples. After this time an exponential growth was detected until 223.00 h, with an optical density of 0.943 and 0.969 in control and pressurized samples respectively. Then was verified an optical density stabilization for both samples types.

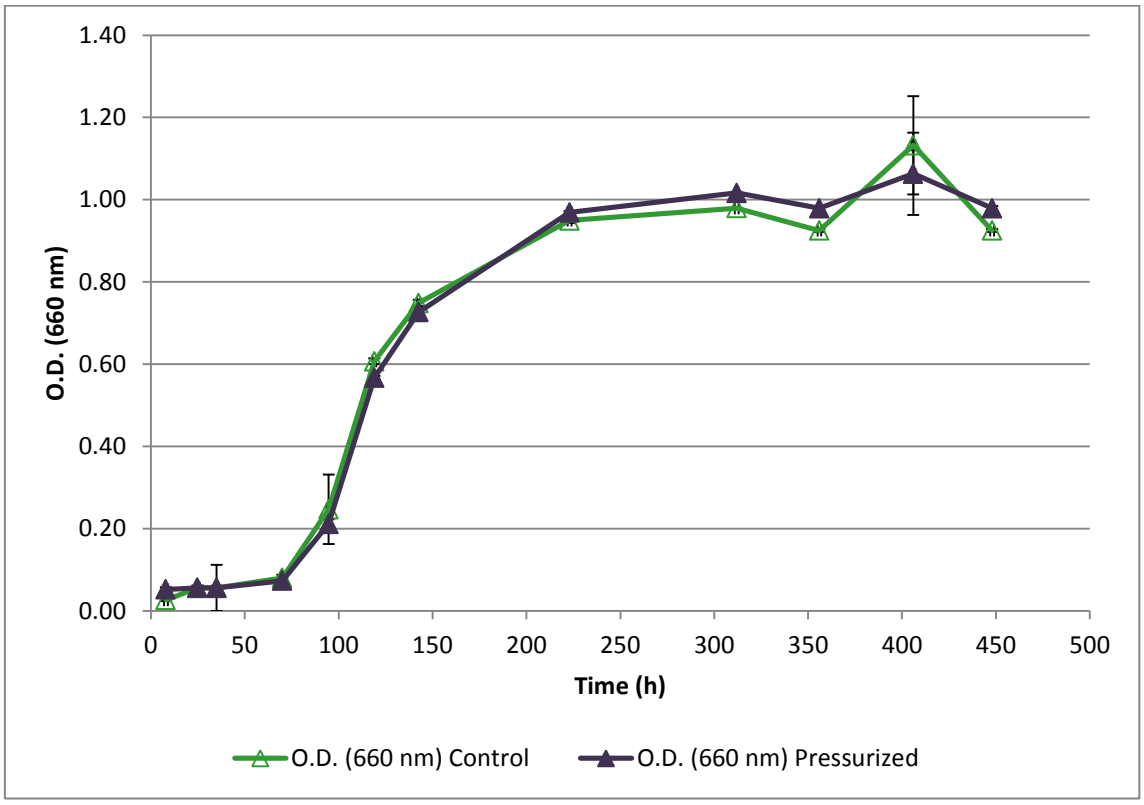


Figure 14 – Optical density (660 nm) in control and pressurized samples, treated at 50 MPa during 8 h

As predicted, the start of a considerable microbial growth coincided with the start of glucose intake. After that, the microbial growth kept increasing, following the glucose consumption, until reaching the stationary phase.

This pressure treatment did not affect the microbial growth compared with control samples. It also did not induce significant alterations in malolactic enzyme and consequently in malolactic fermentation, except the 10.14 h delay. In the case of sugar metabolism it also promoted a delay, but of 24.33 h. Therefore with a 50 MPa during 8 h holding time, the *Oenococcus oeni* was able to conclude successfully the malolactic fermentation, microbial growth and glucose consumption, in about the same time at

atmospheric pressure. This indicates that *Oenococcus oeni* can stand 50 MPa with no significant effect on its metabolic activity.

3.3 100 MPa treatment, during 0.5 h

For a 100 MPa processing, during 0.5 h, Figure 15 illustrates its effect on malolactic fermentation. The L-malic acid concentration in the beginning of the fermentation was 3.61 g/L ($2.69x10^{-2}$ mol/L). For pressurized and control samples, the L-malic acid intake started after 25.97 h of inoculation and finished at 100.72 h with L-malic acid scarcity. As a malolactic fermentation product, the L-lactic acid generation began at 25.97 h and achieved 2.62 g/L ($2.91x10^{-2}$ mol/L) in treated samples and 2.57 g/L ($2.85x10^{-2}$ mol/L) in control samples, at 100.72 h. At this time, the molar ratio was 1.00:1.08 and 1.00:1.06 (L-malic acid:L-lactic acid) for pressurized and control samples respectively. The molar ratio of pressurized samples was very close to the control and no delay was verified, therefore no considerable alterations were verified.

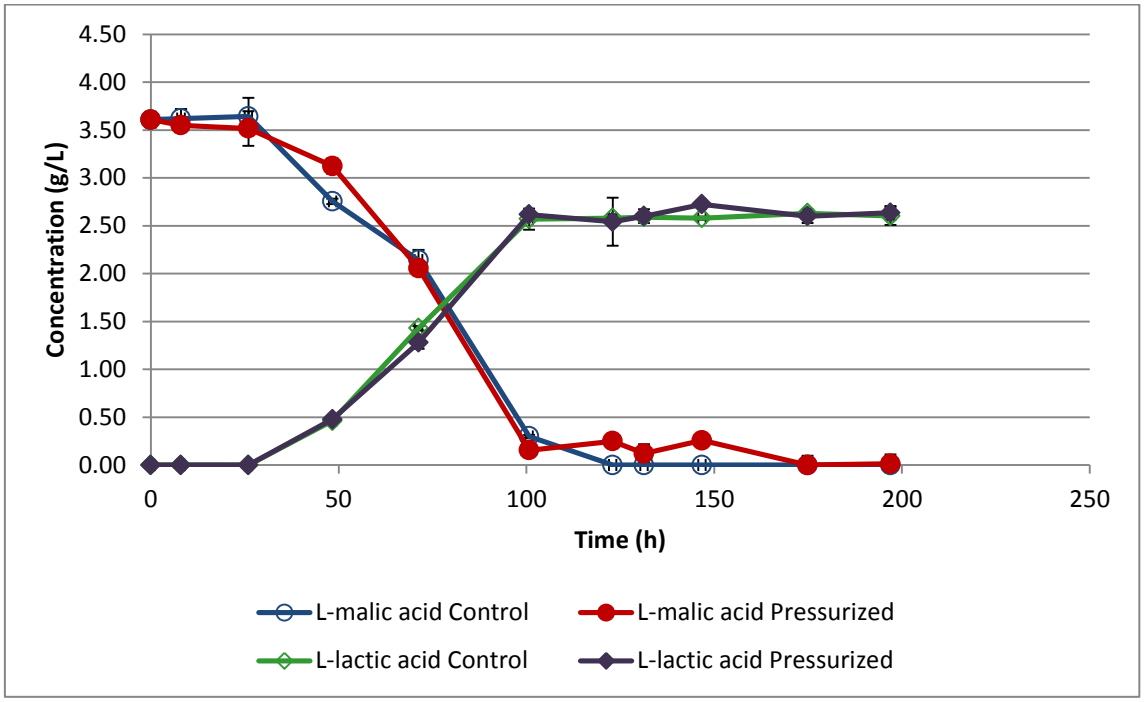


Figure 15 – L-malic acid and L-lactic acid concentration in control and pressurized samples, treated at 100 MPa during 0.5 h

In the case of the sugar metabolism with the same pressure treatment, some alterations were verified (Fig. 16).

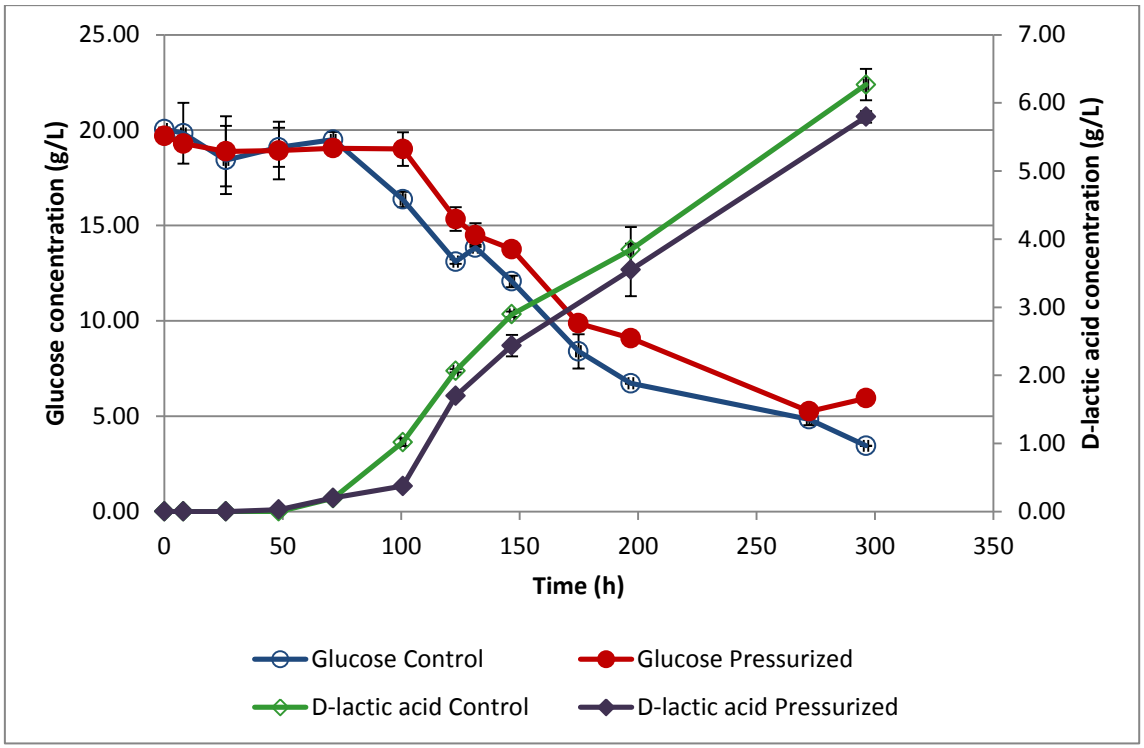


Figure 16 – Glucose and D-lactic acid concentration in control and pressurized samples, treated at 100 MPa during 0.5 h

At the time of the inoculation, the concentration of glucose was 20.05 g/L. Regarding control samples, the glucose intake initiated after 71.27 h, while the pressurized samples only started glucose consumption after 100.72 h. After 296.25 h of inoculation, the glucose concentration was 3.45 g/L in control samples, and 5.95 g/L in pressurized samples. The glucose concentration variation during the experiment was 16.60 g/L (9.21×10^{-2} mol/L) for control samples and 14.10 g/L (7.83×10^{-2} mol/L) for treated samples.

Likewise, the D-lactic acid production started at 71.27 h in control samples and 100.72 h in treated samples. After this time the concentration of this acid kept growing, reaching 5.80 g/L (6.44×10^{-2} mol/L) in pressurized samples and 6.27 g/L (6.96×10^{-2} mol/L) in control samples, at 296.25 h.

The molar ratio between the glucose intake and the D-lactic acid production was 1.00:0.82 and 1.00:0.78 (glucose:D-lactic acid) for pressurized and control samples respectively. These values are very close to each other and small difference can be attributed to experimental errors.

The Figure 16 also suggests that this pressure treatment resulted in a sugar metabolism delay of around 29.45 h, although the microorganisms seem to be able to conclude the glucose consumption.

The effect of this high pressure treatment in optical density (660 nm) is resumed in the Figure 17. At the moment of inoculation the optical density was 0.077. During 71.27 h, no considerable changes were verified in both samples. After that, the optical density began to increase exponentially until the 174.85 h, which was 0.788 in control samples and 0.754 in pressurized samples. At 296.25 h the optical density achieved 0.917 in control samples and 0.793 in pressurized samples.

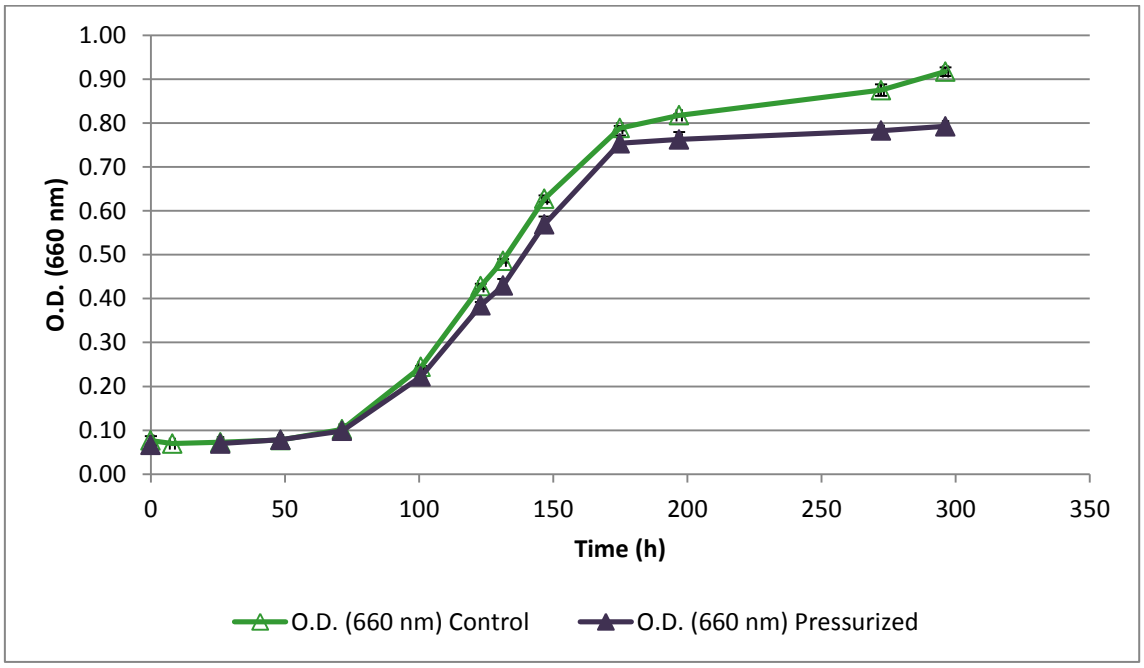


Figure 17 – Optical density (660 nm) in control and pressurized samples, treated at 100 MPa during 0.5 h

Compared with control samples, until **174.85 h** this pressure treatment did not cause significant alterations in microbial growth. However, after this time the biomass production rate was higher in control samples than in pressurized samples.

Summarily, no considerable alterations in malolactic enzyme and consequently in the malolactic fermentation were verified after a 100 MPa treatment, during 0.5 h. In the case of sugar metabolism a delay of **29.45 h** was verified and lower concentration of biomass.

So, at this pressure and holding time, malolactic enzyme is still active to proceed to L-malic acid conversion and *Oenococcus oeni* still able to microbial growth and glucose consumption, in about the same time at atmospheric pressure. This indicates that *Oenococcus oeni* tolerates 100 MPa during 0.5 h, with no significant effect on its metabolic activity.

3.4 100 MPa treatment, during 8h

The L-malic acid intake and L-lactic acid production results, after 100 MPa treatment, during 8 h, are presented in Figure 18. The L-malic acid intake in control samples starts at 25.97 h, with an initial concentration of 3.61 g/L ($2.69x10^{-2}$ mol/L). At 122.97 h, no L-malic acid was detected in control samples. In treated samples, some considerable alterations were found in L-malic acid consumption. Contrarily to control samples, the L-malic consumption only started at 48.35 h and finished at 131.32 h, with all L-malic acid consumed.

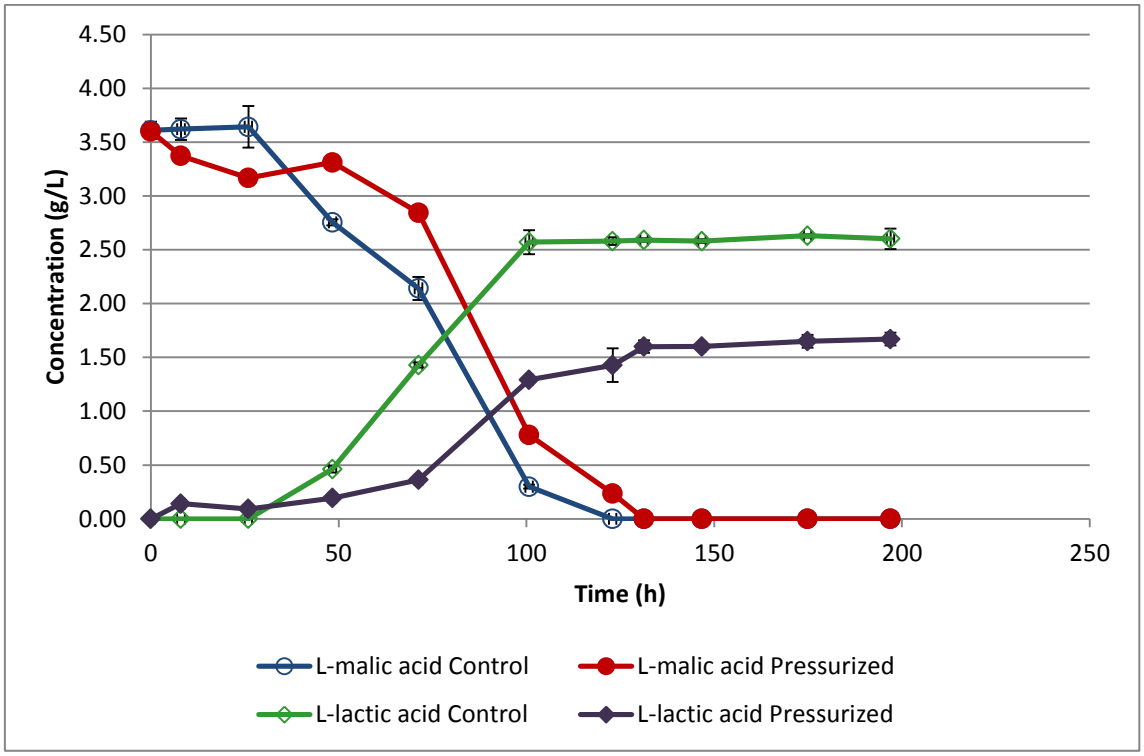


Figure 18 – L-malic acid and L-lactic acid concentration in control and pressurized samples, treated at 100 MPa during 8 h

The L-lactic acid production, as expected, started at 25.97 h for control samples and at 48.35 h for pressurized samples. In control samples at 100.72 h this production stabilized, with 2.57 g/L ($2.85x10^{-2}$ mol/L), while in the pressurized samples it only finished at 131.32 h, but with 1.60 g/L ($1.78x10^{-2}$ mol/L).

In the end of the experiment time, the molar ratio between these two compounds was 1.00:1.06 for control samples and 1.00:0.66 (L-malic acid:L-lactic acid) for treated samples.

With this pressure treatment a considerable difference in molar ratio was achieved. In pressurized samples only about half of the expected L-lactic acid amount was produced. Taking into account this fact and the total consumption of the L-malic acid, another compound might be also produced from this acid or some of the produced L-lactic acid was consumed. The difference between the L-lactic acid in control and pressurized samples, at the final of the experimental time, was 0.97 g/L ($1.08x10^{-2}$ mol/L) (2.57 g/L-1.60g/L).

In addition to this alteration, the treatment also caused a delay in the start in the L-malic acid consumption of 22.38 h.

The glucose concentration and D-lactic acid concentration is resumed in Figure 19.

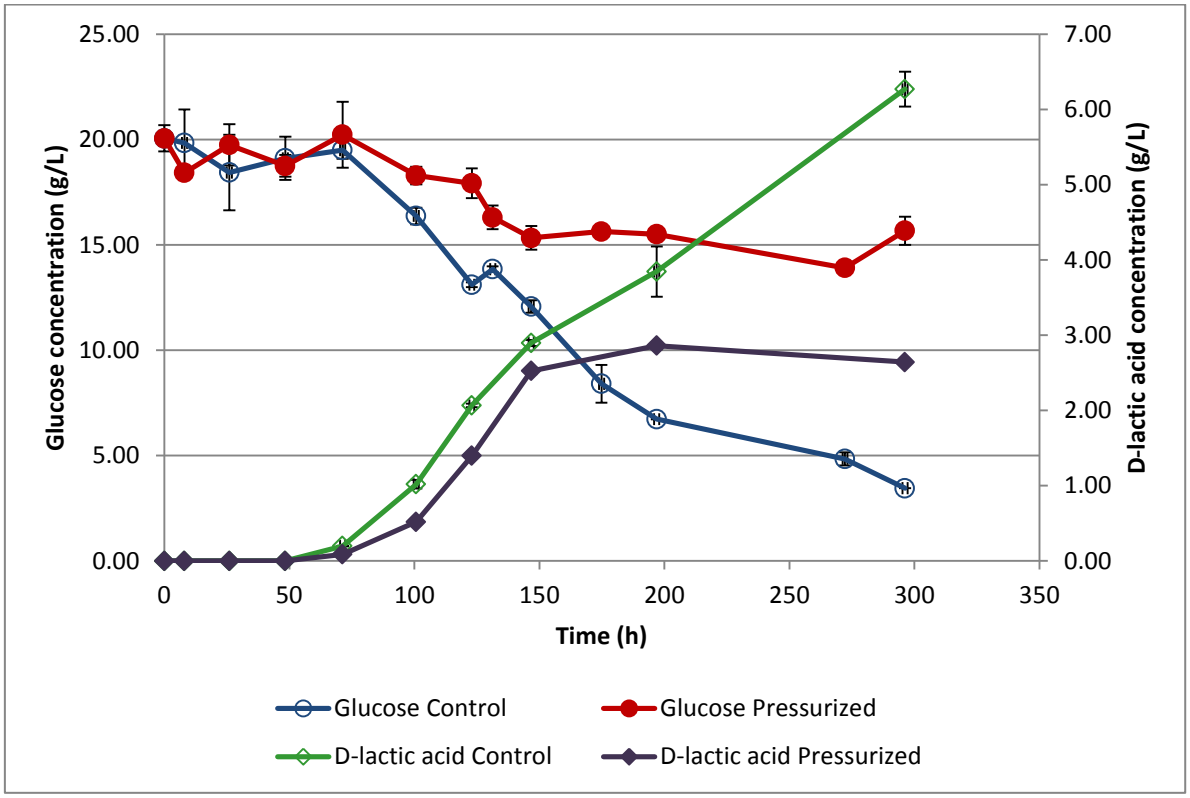


Figure 19 – Glucose and D-lactic acid concentration in control and pressurized samples, treated at 100 MPa during 8 h

For both samples the initial concentration of glucose was 20.05 g/L, and its consumption started after 71.27 h of the inoculation. In control samples the glucose concentration kept decreasing during the experimental time, reaching 3.45 g/L at 296.25, while in pressurized samples it stopped at 146.75 h, with 15.33 g/L of glucose in the medium. The glucose consumption during 296.25 h of experimental time was

16.60 g/L ($9.21x10^{-2}$ mol/L) for control samples and 4.38 g/L ($2.43x10^{-2}$ mol/L) for pressurized samples.

At 71.27 h D-lactic acid production began in control and pressurized samples, but at 146.75 h no more D-lactic acid was formed in treated samples, reaching a final concentration of 2.64 g/L ($2.93x10^{-2}$ mol/L). In the control samples, D-lactic acid concentration continued to grow reaching 6.27 g/L ($6.96x10^{-2}$ mol/L), at 296.25 h.

For this pressure treatment the molar ratio at the end of fermentation was 1.00:0.78 in control samples and 1.00:1.21 (glucose:D-lactic acid) in treated samples.

With this pressure treatment, glucose metabolism exhibited some peculiar differences between treated and control samples.

The first important difference was the high molar ratio obtained in treated samples, between the glucose and D-lactic acid. As previously explained, a lower than 1 molar ratio was acceptable, since glucose may be used for purposes other than product formation. However, a higher than 1 molar ratio only means that some D-lactic acid was being produced from a source, other than glucose. This D-lactic acid may be originary from the malolactic fermentation. During the malolactic fermentation, the pressurized samples showed less 0.97 g/L ($1.08x10^{-2}$ mol/L) of L-lactic acid, than control samples. Assuming the fact that it was formed 0.97 g/L ($1.08x10^{-2}$ mol/L) of D-lactic from L-malic acid, the D-lactic acid produced from glucose in pressurized samples was only 1.67 g/L ($1.85x10^{-2}$ mol/L). Recalculating the molar ratio between the glucose and D-lactic acid, using this concentration of D-lactic acid, the new value was 1:0.76 (glucose:D-lactic acid). This new molar ratio is acceptable and very close to the control value.

Secondly, during the time of glucose intake, its consumption rate was lower in pressurized samples than control samples.

Finally, other important difference was the arrest of the glucose consumption and D-lactic acid production, at 146.75 h after the inoculation. The reason that lead to this stop was not determined, but its occurrence matches exactly with a microbial growth stop, as is demonstrated below, and coincidentally is very close to the L-malic acid exhaustion time.

For this pressure processing, as shown in Figure 20, the optical density was 0.077, at the time of inoculation. At 71.27 h this measure started increasing for both types of samples. In the case of control samples this growth only slowed down at 174.85 h, with

an optical density of 0.788, while on pressurized samples it finished at 146.75 h (coinciding with glucose intake stop), with an optical density of 0.494.

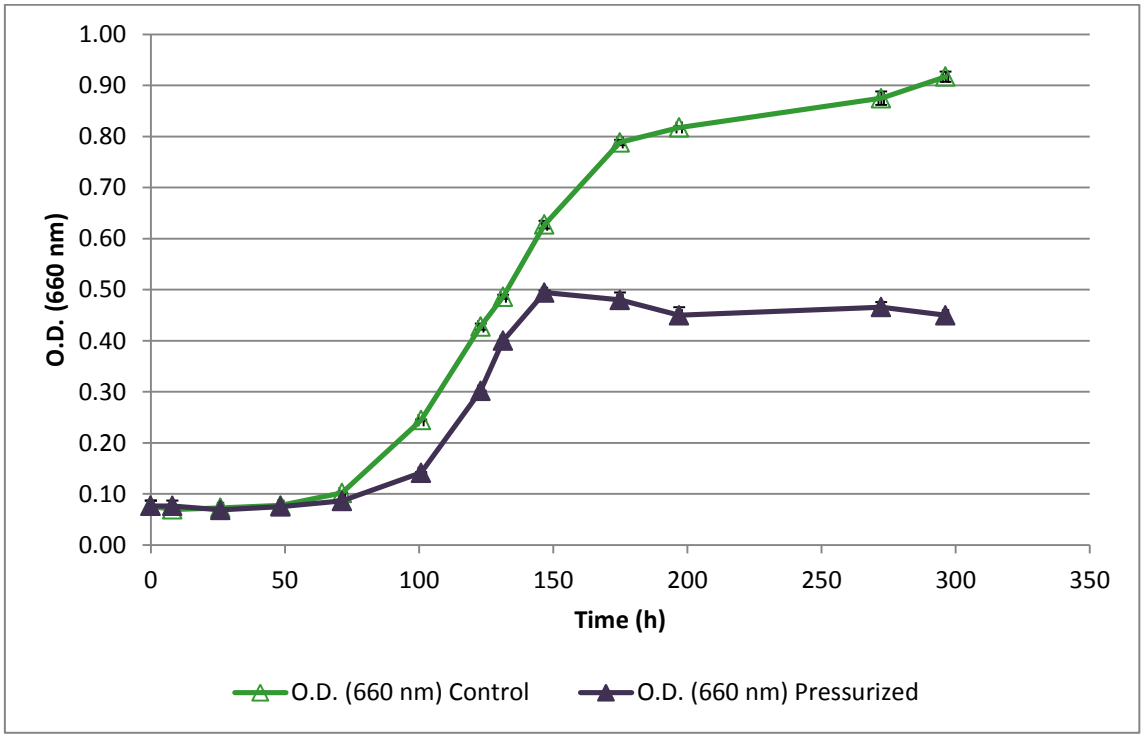


Figure 20 – Optical density (660 nm) in control and pressurized samples, treated at 100 MPa during 8 h

As expected, the microbial growth was also affected by the alterations in the sugar metabolism. The interval of considerable microbial growth was correspondent to the interval of glucose intake and D-lactic acid production. At the end of this interval, as in sugar metabolism, the biomass production stopped completely. After that the optical density in pressurized samples did not show a smooth increase, like in control samples. This fact suggests a complete inactivation, which cause was not determined. However, a colony-formers plate count is necessary to clarify the reason of this effect.

Therefore, the pressure treatment of 100 MPa, during 8 h, causes alterations in malolactic enzyme and consequently in the products of the L-malic acid. A lower amount of L-lactic acid was detected in the pressurized samples than in the control. It was proposed the production of D-lactic acid from L-malic acid to explain that fact, thus implying a change of specificity in the malolactic enzyme. This was caused by the high pressure treatment, making it able to produce the two stereoisomers of the lactic acid, from L-malic acid. However to find more proofs of this fact, it is necessary proceed to same experiment using L-malic acid labeled with radioactive isotopes.

In sugar metabolism, in addition to the lower glucose rate consumption found, an unexpected stop of glucose consumption and microbial growth was detected. The cause of this phenomenon was not determined. However its determination is fundamental to have a better elucidation of the effect of this pressure treatment in *Oenococcus oeni* sugar metabolism.

3.5 100 MPa treatment, during 60 h

The Figure 21 resumes the effect of 100 MPa treatment, during 60 h in malolactic fermentation.

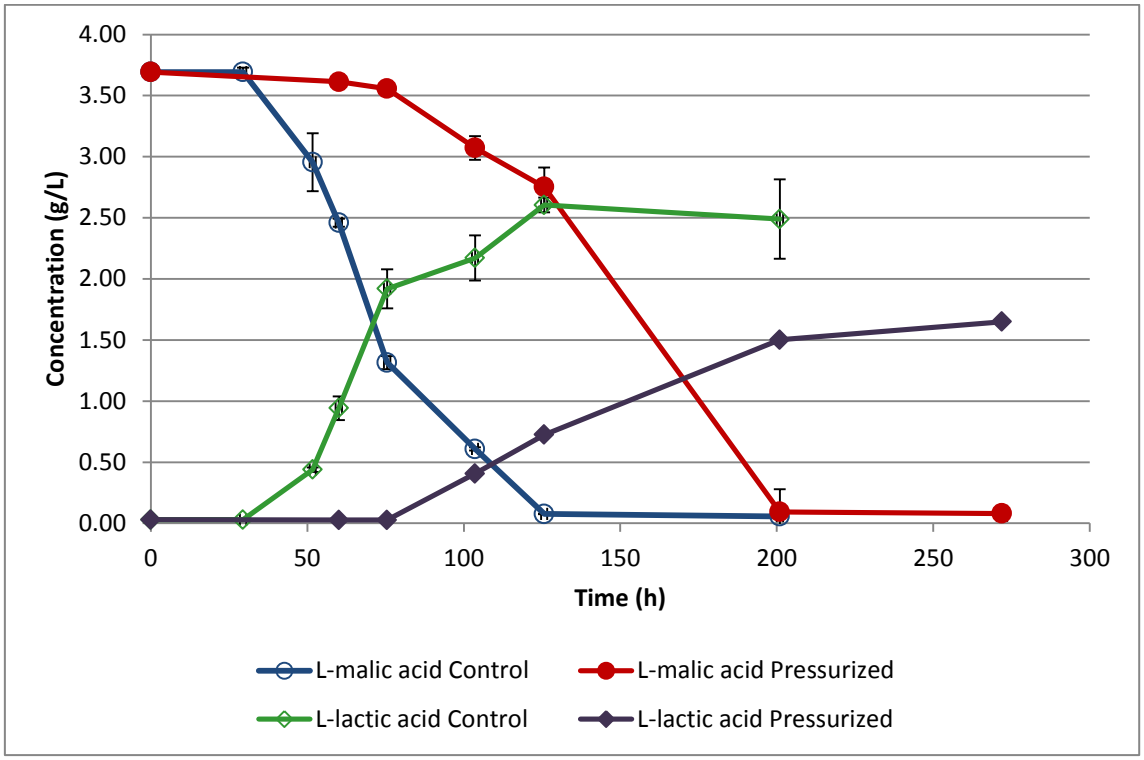


Figure 21 – L-malic acid and L-lactic acid concentration in control and pressurized samples, treated at 100 MPa during 60 h

The initial concentration of L-malic acid in this experiment was 3.69 g/L ($2.75x10^{-2}$ mol/L). In the control samples a decrease of the L-malic concentration began at 29.45 h and finished at 125.83 h, with all L-malic acid consumed. In pressurized samples the L-malic acid consumption only started at 75.48 h and finished at 200.98 h, with no L-malic acid presented in the medium.

The production of the L-lactic acid started at 29.45 h and ended at 125.83 h in control samples, while in pressurized samples started at 75.48 h and ended at 200.98 h, as predicted. After 200.98 h a smooth increase was verified in pressurized samples, however, this increase was related to some experimental error, because the L-malic acid exhaustion was at 200.98 h. The final concentration was 2.60 g/L in control samples ($2.89x10^{-2}$ mol/L) and was 1.62 g/L ($1.80x10^{-2}$ mol/L) in treated samples.

The final molar ratio between L-malic acid and L-lactic acid was 1.00:1.05 for control samples and 1.00:0.65 (L-malic acid:L-lactic acid) for pressurized samples.

Compared with 8 h treatment at same pressure, this pressurization induced a higher delay in L-malic acid consumption starts (46.03 h) and a similar difference in molar ratio was also achieved. Likewise, this means that another compound was also produced from L-malic acid or some L-lactic acid produced was consumed. The difference between the L-lactic acid in control and pressurized samples, at the final of experimental time, was 0.98 g/L ($1.09x10^{-2}$ mol/L) (2.60 g/L-1.62g/L).

The Figure 22 displays the glucose and D-lactic acid concentration, during this high pressure treatment. Before the treatment, the glucose concentration was 19.95 g/L. In control samples the degradation of the glucose started at 68.03 h, while in pressurized samples at 125.57 h. It was verified a continuing glucose degradation, during all the experiment in control samples, reaching a concentration of 2.80 g/L, at 292.25 h. On other hand, the glucose degradation finished in pressurized samples at 208.25 h, with a concentration of 15.33 g/L.

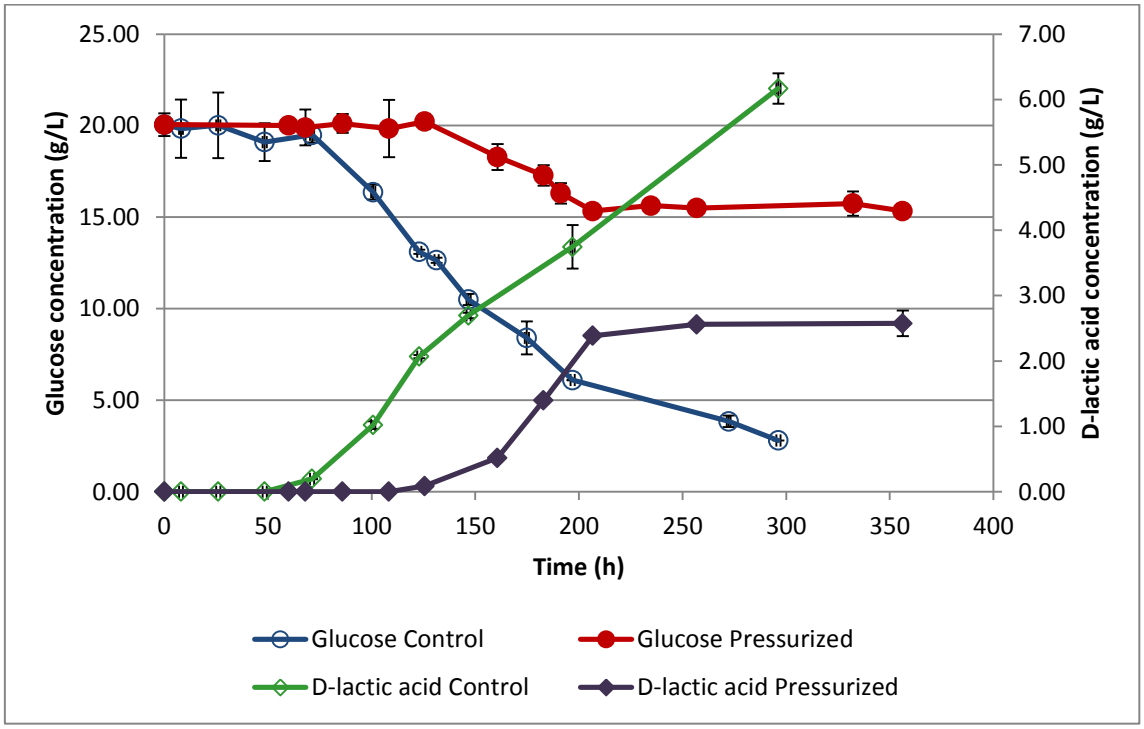


Figure 22 – Glucose and D-lactic acid concentration in control and pressurized samples, treated at 100 MPa during 60 h

The D-lactic acid production was started at same time of its respectively glucose degradation and for pressurized samples also finished at the same time. The final concentration was 2.57 g/L in treated samples and 6.17 g/L in control samples.

At the end of the experimental time were consumed 17.15 g/L ($9.52x10^{-2}$ mol/L) of glucose and produced 6.17 g/L ($6.85x10^{-2}$ mol/L) of D-lactic acid, in control samples. In pressurized were consumed 4.62 g/L ($2.56x10^{-2}$ mol/L) of glucose and produced 2.57 g/L ($2.85x10^{-2}$ mol/L) of D-lactic acid. Therefore, the molar ratio was 1.00:0.72 and 1.00:1.11 (glucose:D-lactic acid) in control and pressurized samples respectively.

Like in the previously treatment, this pressurization also promote interesting alterations in sugar metabolism.

Firstly, analyzing the higher molar ratio obtained in pressurized samples, some D-lactic acid was being produced from a source, other than glucose.

From malolactic fermentation the pressurized samples were less 0.98 g/L ($1.09x10^{-2}$ mol/L) of L-lactic acid, than control samples. Assuming again the fact that it was formed 0.98 g/L ($1.09x10^{-2}$ mol/L) of D-lactic from L-malic acid, the D-lactic acid produced from glucose in pressurized samples was only 1.59 g/L ($1.77x10^{-2}$ mol/L). Recalculating the molar ratio between the glucose and D-lactic acid, using this concentration of D-lactic acid, the new value was 1.00:0.69 (glucose:D-lactic acid). This new molar ratio is acceptable and very close to the control value. So, this D-lactic acid may became from the malolactic fermentation.

The pressure treatment also induced a lag of 57.54 h in the start of the glucose intake and an interruption of the glucose consumption at 208.25 h, like occurred in the 100 MPa pressurization during 8 h.

The Figure 23 illustrates microbial growth during experimental time. The optical density at the beginning of the fermentation was 0.077 and started increasing at 68.03 h in control samples, while in processed samples only at 125.57 h. After that, it was detected an exponential growth in both samples. The microbial growth started a smooth increase in control samples at 174.85 h, with an optical density of 0.788, while in pressurized samples at 206.75 h, with an optical density of 0.444.

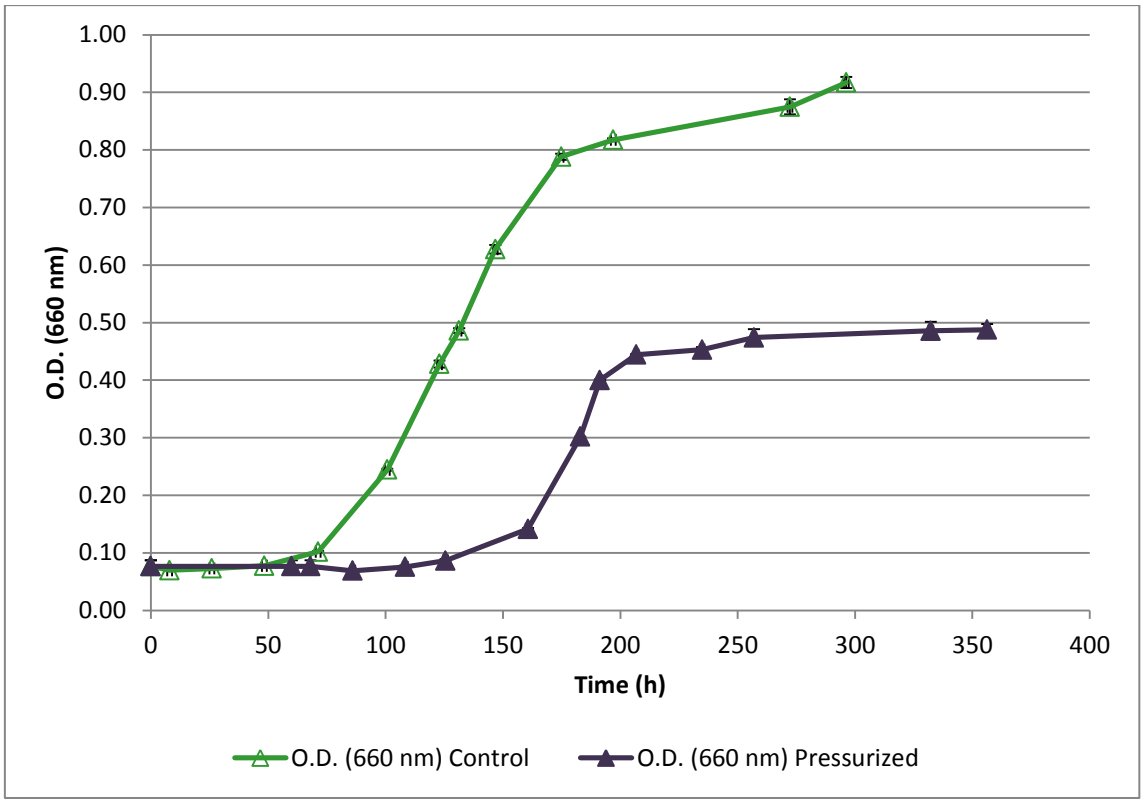


Figure 23 – Optical density (660 nm) in control and pressurized samples, treated at 100 MPa during 60 h

This pressure treatment, with a same pressure and a longer time than the last presented, had the same effect, but with longer delays.

3.6 300 MPa treatment, during 0.5 h

The Figure 24 resumes the effect on malolactic fermentation of 300 MPa treatment, during 0.5 h. At the instant of the inoculation the L-malic acid concentration was 3.61 g/L and as expected in control sample, it was started a considering consumption at 25.97 h and finished at 122.97 h. However in pressurized samples a smooth variation was obtained after 196.95 h, which was 3.02 g/L of L-malic acid concentration.

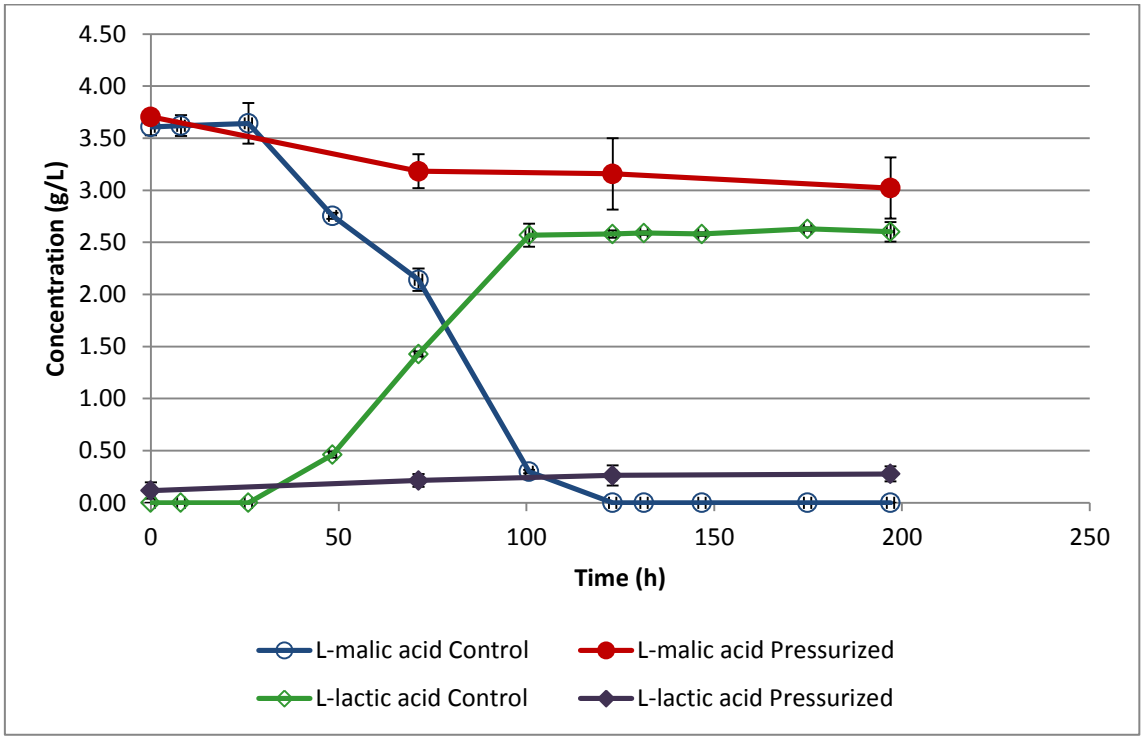


Figure 24 – L-malic acid and L-lactic acid concentration in control and pressurized samples, treated at 300 MPa during 0.5 h

For the L-lactic acid, the correspondent type of alterations was verified, with an L-lactic acid production in control samples after 26.00 h of the inoculation, reaching 2.57 g/L, at 100.72 h of fermentation. On other hand the L-lactic acid concentration, in pressurized samples was not varies significantly, with a 0.277 g/L, at 196.95 h.

The very smooth variation of the L-malic acid and L-lactic concentrations reveals a presence of some active malolactic enzyme in the medium. As is explained below, this treatment resulted in total inactivation of the inoculum microorganisms. However, a portion of the malolactic enzyme from these inactivated microorganisms seems to remain active in the medium. Therefore, this pressure treatment seems not being able to inactivate completely the malolactic enzyme.

The total experimented time was not enough to understand if this small amount of active malolactic enzyme was able to complete the malolactic fermentation.

The effect of this process in the sugar metabolism is resumed in the Figure 25. In the case of the control samples, the glucose degradation started at 71.27 h, with an initial concentration of 20.05 g/L and a final concentration of 3.45 g/L, at 296.25 h. The D-lactic acid production, in those samples, started at 71.27 h and after 296.25 h of the inoculation, the concentration was 6.27 g/L.

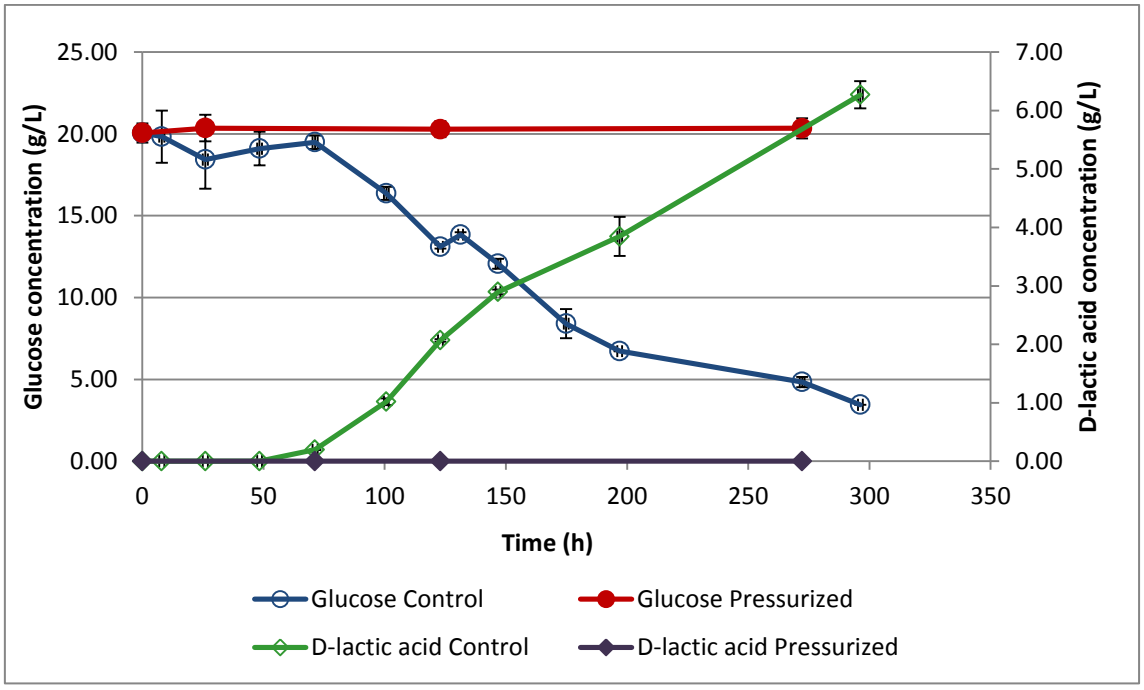


Figure 25 – Glucose and D-lactic acid concentration in control and pressurized samples, treated at 300 MPa during 0.5 h

Contrarily, in processed samples was not observed glucose variation during the experiment, with an initial concentration of 20.05 g/L and a final of 20.34 g/L, at 296.25 h. For the D-lactic acid, it also was not detected any formation during 296.25 h.

It was not verified a considerable variation of the glucose and D-lactic acid concentrations. Therefore these results suggest a complete inactivation of the microorganism presents in the medium, resulted from 300 MPa treatment during 0.5 h. However the next Figure (Fig. 26) clarifies better this fact.

The effect of this treatment on microbial growth is represented in the Figure 26. In the case of the control samples, the initial optical density was 0.077 and at 71.27 h, initiates an exponential growth until 174.85 h, with an optical density of 0.788. Then follow a smooth increase, reaching 0.917 at 296.25 h.

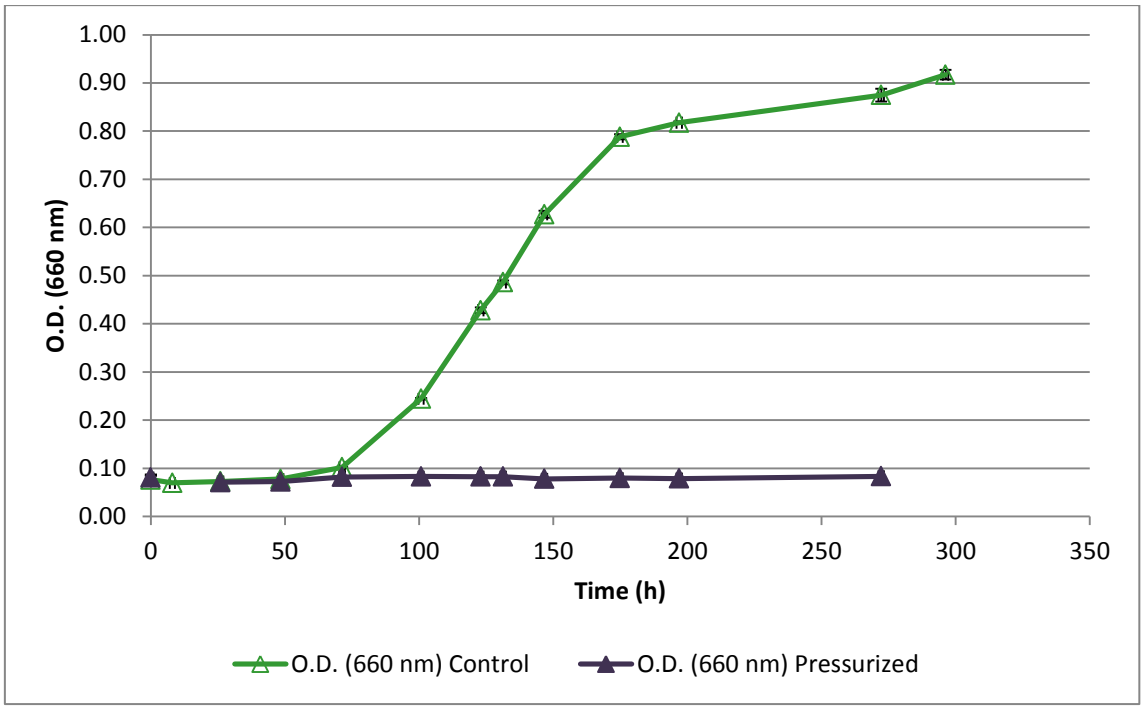


Figure 26 – Optical density (660 nm) in control and pressurized samples, treated at 300 MPa during 0.5 h

Contrary to the control samples, the optical density (660 nm) of the pressurized samples remained stable during all experiment, with 0.077 at the beginning and 0.083 at 272.25 h.

This pressure treatment resulted in a non-considerable variation of the optical density and glucose consumption. It means that no cell growth occurred during the experimented time. In other words the pressure treatment of 300 MPa, during 0.5 h resulted in a complete inactivation of the microorganisms. However the malolactic enzyme seems still with some activity. These results have some biotechnological applications to proceed to *Oenococcus oeni* inactivation, preserving the medium (wine) and for malolactic enzyme extraction, if proved the activity of this enzyme after this high pressure treatment.

CHAPTER IV

CONCLUSION AND FUTURE WORK

As described previously the technology of high hydrostatic pressure has great potential to explore new and promising applications in biotechnology. This variable affects, like temperature, microbial growth and metabolism due to its impacts on different cellular constituents. However, the study of the effects of pressure in fermentations is still very scarce. At this moment, there are only a few articles in this area. They demonstrate that the stress response of microorganisms under pressure can lead to an increased fermentation rate, product yield or a different metabolism profile.

This work studied the effect of this important natural variable in the *Oenococus oeni* (malolactic bacteria) metabolism, in order to improve the processes involving this bacteria, for example reducing the process time, increasing yields, or producing new compounds.

As expected, high pressure affects the *Oenococcus oeni* metabolism and its effect varies according to pressure and holding time.

Lower pressures, with higher holding times (50 MPa, during 8h) and higher pressures, with lower holding times (100 MPa, during 0.5 h) don't affect significantly the L-malic conversion to L-lactic acid, but promote a small delay in glucose metabolism start.

On the other hand, higher pressures with longer holding times (100 MPa during 8 h and 60 h) seem to cause alterations in the malolactic enzyme, leading to the production of lower L-lactic acid amounts than it was previously expected. The stoichiometric calculations indicate that it can be being produced D-lactic acid from L-malic acid. These treatments also promote a delay in glucose consumption start, and a stop of microbial growth and glucose intake at the time of L-malic acid exhaustion, without determined reason. The observed delay was higher according the pressure and holding time.

Pressures above 300 MPa with holding times higher than 0.5 h lead to a complete microbial inactivation of *Oenococcus oeni*. However, with these conditions, the malolactic enzyme seems to show some residual activity.

This work was a preliminary study of the effect of pressure in *Oenococcus oeni* metabolism and it proves the possibility of this bacteria being able to proceed to a fermentation after a pressure treatment, with a modified metabolism, according to the pressure and the holding time used. The main modification obtained is the production of L-lactic acid and D-lactic acid from L-malic acid, caused by the modification of the malolactic enzyme specificity. Therefore, this work reveals a possible impact of high

pressure on enzymes, leading to changes of specificity. These results have substantial and immediate impact on the possibilities of improvement of biocatalized processes, with a wide range of unstudied paths.

However, to complement this concrete study, much work remains to develop. It is necessary to prove the formation of D-lactic acid, from L-malic acid, by *Oenococcus oeni* malolactic enzyme, probably using L-malic acid labeled with radioactive isotopes. It is also necessary to determine the reason that leads to glucose consumption and microbial growth arrest. Beyond that, complete fermentations under pressure may originate interesting results. Beside the *Oenococcus oeni* metabolism, these results also suggest studies of the effect of the high pressure in malolactic enzyme concretely, using a purified enzyme extract.

Notwithstanding, there is still a long path to be taken before high pressure can be used to improve fermentations and biocatalyzed processes

CHAPTER V

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CHAPTER VI

APPENDICES

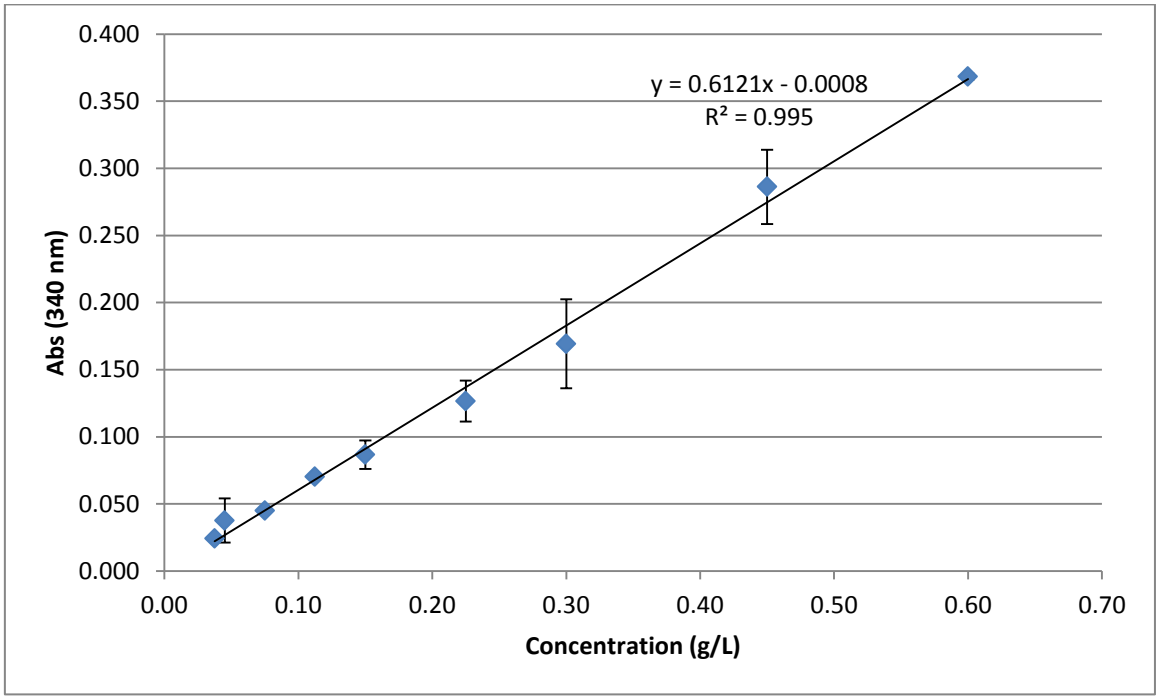

Figure 27 – L-malic acid calibration curve and equation

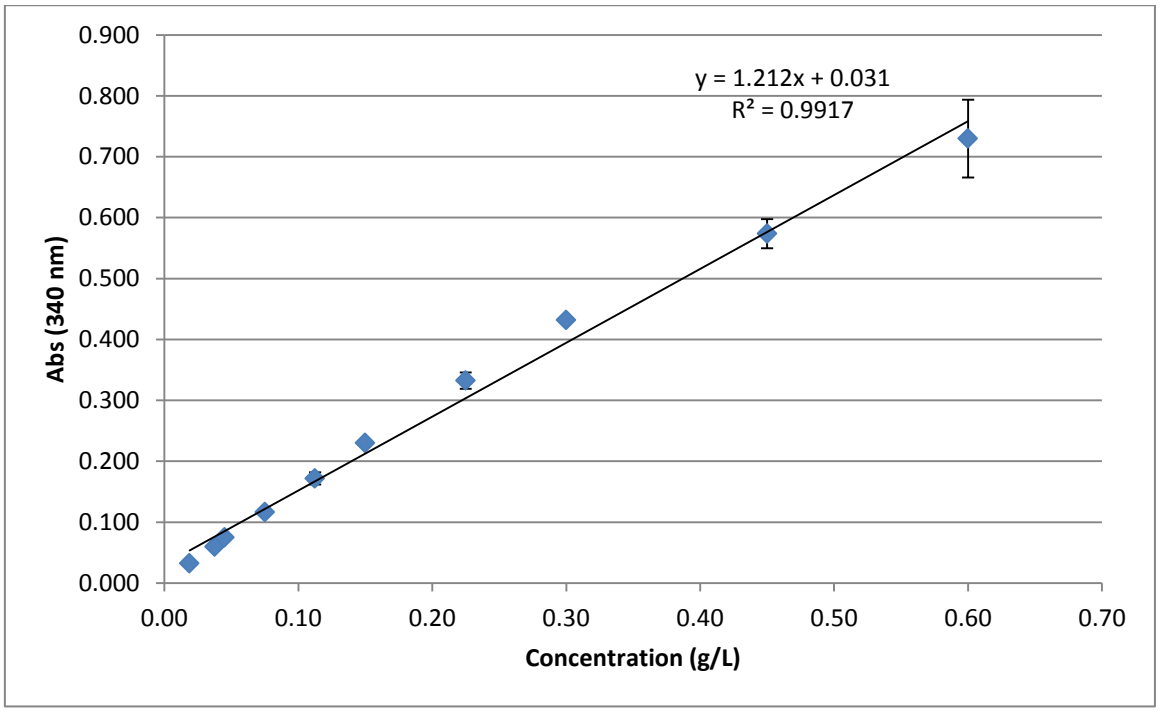

Figure 28 – L-lactic acid calibration curve and equation

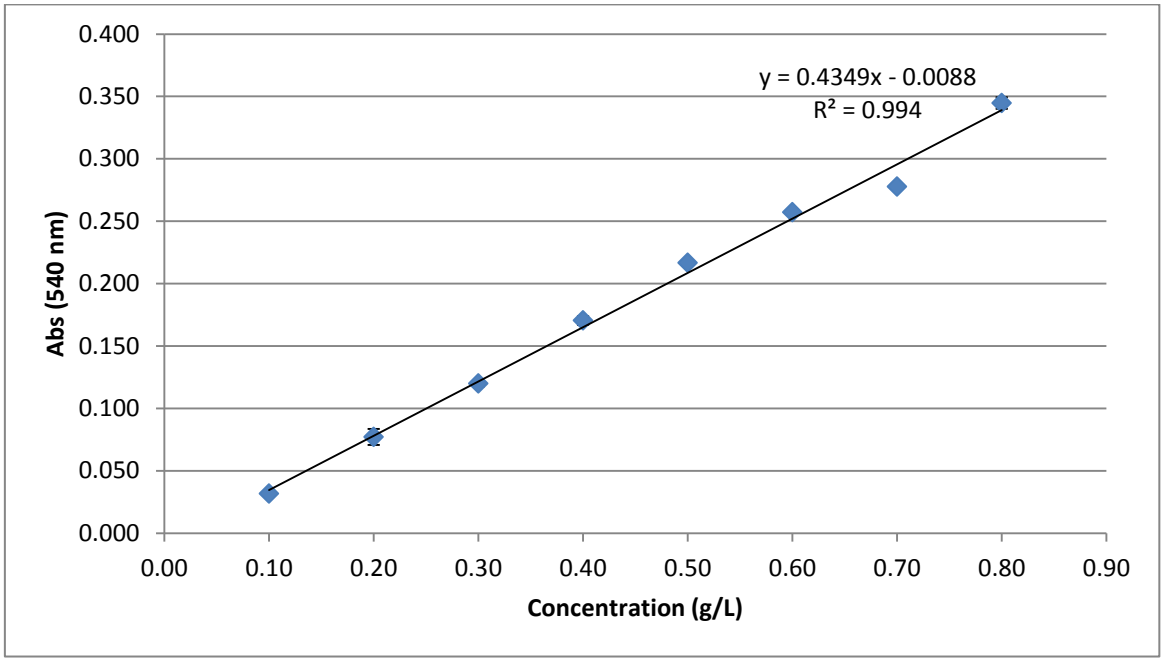

Figure 29 – Glucose calibration curve and equation

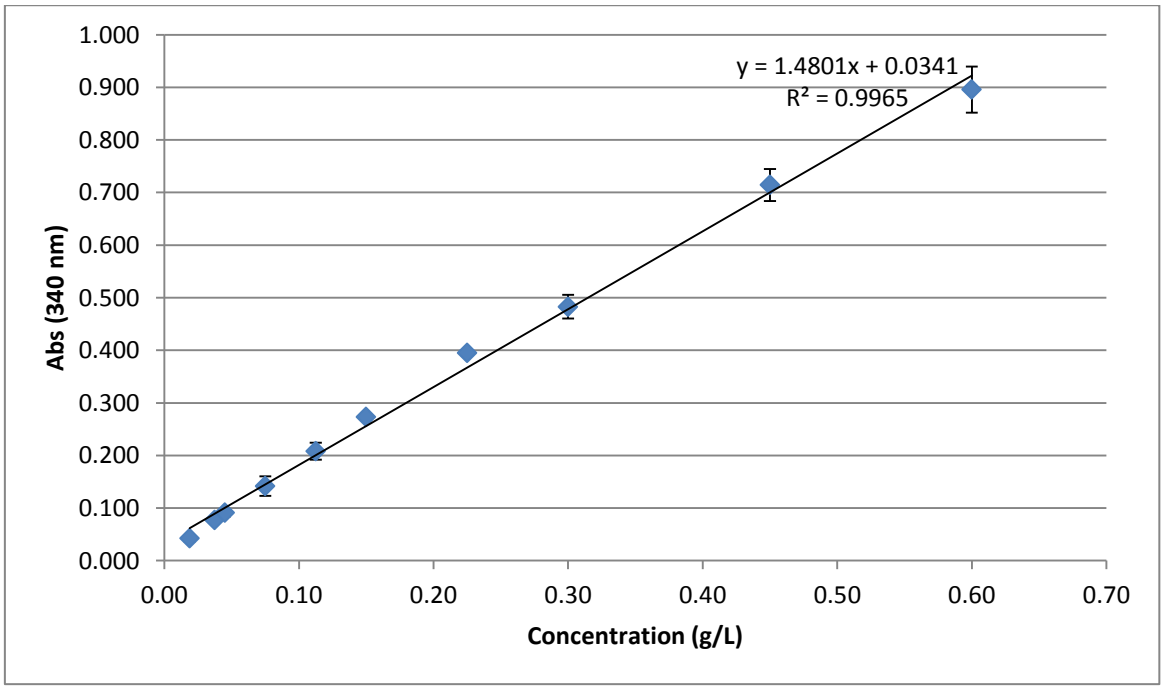

Figure 30 – D-lactic acid calibration curve and equation